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STUDIES ON TUBERCULOSIS

By

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AND CLAUDE E. FORKNER, M.D.

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BY FLORENCE R. SABIN, M.D., CHARLES A. DOAN, M.D., AND CLAUDE E. FORKNER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 1 TO 10

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CHAPTER I

REACTION OF THE CONNECTIVE TISSUES OF THE NORMAL RABBIT TO LIPOIDS FROM THE TUBERCLE BACILLUS, STRAIN H-37

By FLORENCE R. SABIN, M.D., CHARLES A. DOAN, M.D., and CLAUDE E. FORKNER, M.D.

Plates 1 to 7

INTRODUCTION

This series of studies represents an effort to analyze how far each one of the manifestations of tuberculosis can be related to specific chemical substances isolated from the tubercle bacillus. The factor considered here is the production of new tissue, with the formation of the tubercle as the most significant element. We shall show that the substances from the tubercle bacillus which give typical tubercular tissue are limited to the lipoid fractions. Epithelioid cells are not produced in the normal animal by the soluble tuberculo-proteins or by the polysaccharide derivatives. The fractions used were from the human strain of tubercle bacilli, H-37; but comparative studies are being made with fractions from the bovine and avian strains as well as from the timothy grass bacillus (1). These studies with the lipoids have been made for the most part in the normal rabbit.

During the years since 1891, when Koch (2) first reported his observations on the effect of injecting killed tubercle bacilli, many studies have been made of the changes produced in tissues by dead tubercle bacilli and by lipoids extracted from them; the reactions produced by other lipoids extracted from various bacilli and from certain of the organs of the body, such as the liver, have also been described. This literature was analyzed in our first report on the biological reactions to fractions from the tubercle bacillus (Sabin and Doan, 3). Weyl (4) was the first to use chemical extracts from the tubercle bacillus in such work. As a result of many studies, the effect of any lipoid injected into the tissues has come to be considered as belonging to the common type of the so-called foreign body reaction (Morse and Stott, 5; Ray and Shipman, 6). The individual differences in results

are difficult to evaluate both on account of an inadequate chemical separation of the different types of lipoids and because of obscurities in the analysis of the complex effects in the tissues. In general they indicate that the tubercle, the lesion which, in association with the tubercle bacillus, is characteristic of the disease, can be produced either by dead tubercle bacilli or by lipoids extracted from them.

The chemical fractions have been made available through a plan for cooperative research organized by the Research Committee of the National Tuberculosis Association (William Charles White, Bureau of Hygiene, Washington, D. C., Chairman). Acid-fast bacilli of standard strains have been grown in large quantity under uniform conditions, on Long's synthetic medium, by H. K. Mulford Company and by Parke, Davis and Company. A complete bibliography of all the work under this Committee has been published recently by the National Tuberculosis Association (1). Chemical analyses of bacilli and filtrate have been made by Dr. Treat B. Johnson and Dr. R. J. Anderson and their associates at the Sterling Chemistry Laboratory of Yale University, and of the filtrate by Dr. E. R. Long and Dr. F. B. Seibert, Department of Pathology, University of Chicago. So comprehensive has been the plan of this work that chemical products of uniform standard, certainly products that can be used for comparable biological studies, are now becoming available in considerable quantities from each of the several members of the acid-fast group of bacteria.

In these papers we shall use the terms "tuberculous" and "tubercular" in the senses recommended by the National Tuberculosis Association. "Tuberculous" tissue and tubercle will be understood to mean lesions produced in the actual disease, and hence under the influence of the tubercle bacillus; while "tubercular" tissue and tubercle-like structures will be used to describe lesions resembling those of the disease, but produced by the chemical products from the bacillus. The need for the term "tubercular" is an indication that lesions resembling tubercles—more specifically, diffuse and massive collections of epithelioid cells—occur in other pathological conditions than those set up by the tubercle bacillus. In this paper we shall refer to lesions produced by chemical fractions from the tubercle bacillus, involving epithelioid cells and giant cells of the Langhans type, as characteristic "tubercular" lesions whether the epithelioid cells are diffusely scattered in a tubercular granulation tissue, or are in the form of compact clumps or tubercle-like structures. All the other connective tissue reactions found in the disease and reproduced by the chemical fractions, such as the draining of leucocytes from blood to tissues, increase in cell division or in phagocytic activity of clasmatocytes, foreign body giant cells, increase in undifferentiated fixed connective tissue cells, or in fibroblasts, new growth of blood vessels, increase in lymphocytes either from the blood or from a local development in the tissues, will be spoken of as general or non-specific lesions.

During recent years, the development of methods for studying living cells by the supravital technique and by tissue culture has given new

criteria for estimating and differentiating biological reactions. These studies have made it possible to detect epithelioid cells, the essential cellular units of the tubercle, in fresh tissues, even when they are few and scattered, with more certainty than in sections. In a study of giant cells with the supravital technique, reported in this monograph (7), evidence is cited to show that the so-called Langhans giant cell is a direct derivative of a single epithelioid cell, whereas the foreign body giant cell (8) results from the fusion of monocytes, epithelioid cells, and possibly other mononuclear cells. The name epithelioid giant cell has therefore been suggested for the former type to indicate that it is a multinuclear epithelioid cell. It has been shown that in the disease tuberculosis, and around foreign bodies as well, both types of giant cells occur, but in varying proportions according to the type of stimulus. As is well known, the epithelioid giant cell predominates in tuberculosis.

It is feasible therefore to stress the production of epithelioid cells and epithelioid giant cells, in contrast to other mononuclear and multinuclear forms, as perhaps the most significant factor in the pathology of tuberculosis.

REACTION TO THE PHOSPHATIDE A-3

In the analysis of the lipoids made by Anderson, a phosphatide designated by him A-3 has proved to have the most significant biological properties. It not only produces tubercular tissue in greatest amount, but it may act as an antigen; in the one instance its effect is to increase the lesions of the disease, in the other it may tend to reduce them. The specific cellular stimulant in the phosphatide is a saturated fatty acid.

From Table I it will be seen that we have received from Dr. Anderson[1] (9) four lipoid partitions, a phosphatide A-3, a so-called purified wax, a soft wax, and glycerides. Their respective solubilities are shown in the table. The total lipoids Dr. Anderson reports as about 24 per cent of the dry bacilli, Strain H-37. The waxes comprise 46 per cent (11 per cent of the dry bacilli); and the phosphatide 20 per cent (5

[1] Beside the references of papers already published, Dr. Anderson is now writing a complete account of his chemical analyses up to the present time.

TABLE I

Effects of Tuberculo-Lipoids Prepared by Anderson Compared with Effects of Tubercle Bacilli

Living Tubercle Bacilli

1. Leucocytes, neutrophilic (acute, 24 hours).
2. Clasmatocytes (phagocytizing leucocytes).
3. TUBERCLES (monocytes, epithelioids, giant cells).
4. Lymphocytes (chronic).

Dead Tubercle Bacilli

1. Leucocytes, neutrophilic (acute, 24 hours).
2. Clasmatocytes (phagocytizing leucocytes).
3. TUBERCLES (monocytes, epithelioids, giant cells).
4. Lymphocytes (chronic).

} Lacks power of extension of lesions.

Effects of Intraperitoneal Injections of Chemical Fractions from Tubercle Bacilli, Strain H-37

Tuberculo-proteins	Lipoids				Polysaccharides
	Acetone-insoluble			Acetone-soluble	
	Alcohol-ether-soluble	Chloroform-soluble		Alcohol-ether-soluble	
Leucocytes, neutrophilic Clasmatocytes phagocytizing leucocytes	*Phosphatide A-3* 1. Leucocytes, neutrophilic 2. Clasmatocytes 3. Tubercular tissue ++++ 4. Lymphocytes	*Purified wax* 1. Leucocytes, neutrophilic 2. Clasmatocytes 3. Tubercle-like structures ++ 4. Increased connective tissue	*Soft wax* 1. Leucocytes, neutrophilic 2. Clasmatocytes 3. Tubercle-like structures +++	*Glycerides* Not tested	Leucocytes, neutrophilic Clasmatocytes phagocytizing leucocytes

Phosphatide A-3	*Purified wax*	*Soft wax*	*Glycerides*	
	A. Unsaponifiable substance Increased connective tissue		A. Fatty acid IV Few epithelioid cells	B. Fatty acid V Epithelioid cells +
A. Oleic acid B. Palmitic acid C. Glycerophosphoric acid (all nonspecific connective tissue) D. Glucose E. Sugar acid				
F. Fatty acid I 1. Leucocytes 2. Clasmatocytes 3. Tubercle-like structures ++++ 4. Lymphocytes	B. Fatty acid II 1. Leucocytes 2. Clasmatocytes 3. Tubercle-like structures ++ 4. Increased connective tissue	A. Fatty acid III 1. Leucocytes 2. Clasmatocytes 3. Tubercle-like structures +++	*Separated by fractional distillation* Tuberculo-stearic acid Optically inactive Negative for epithelioid cells	Phthioic acid Optically active Epithelioid cells + Increased connective tissue

per cent of the dry bacilli). In the lower half of the table are listed the chemical products derived from the four major fractions, so that it is possible to compare the biological activity of the larger molecules of the original fractions with the active principle for the formation of epithelioid cells.

Of all the lipoids tested, the phosphatide A-3 and the saturated fatty acid derived from it are the most potent in producing tubercular tissue. The other lipoid fractions, purified wax, soft wax, and glycerides, contain active fatty acids to a less degree.

In any study of the action of the lipoid fractions with respect to their power to produce lesions, the route of injection is important. At the present time we shall discuss the effects of the intraperitoneal route only; the effects of intravenous (10) and subcutaneous injections, also significant, require separate presentation.

The lipoids produce a marked new growth of general connective tissue as well as tubercular granulation tissue and tubercle-like structures; only the development of epithelioid cells is stressed in the table, and is designated by the words "tubercular tissue," or "epithelioid cells," or "giant cells" (the Langhans or rosette giant cells, derived from the epithelioid cell).

The criteria which have been met for the designation of any reaction as "specific," or positive, are as follows:

1. The maturation and multiplication of new monocytes. The epithelioid cell is not the immediate reaction to active chemical substances. Preceding its appearance there is a maturation of new young monocytes, identified with the supravital technique by their characteristic reaction to neutral red and demonstrated in sections in division. An active cell division is an important feature, since, though monocytes occur normally in considerable numbers in the omentum of certain rabbits, the specific lesions produced involve an increase in these cells.

2. The occurrence of epithelioid cells by the 4th day.

3. The presence of epithelioid cells, after from seven to twelve daily doses, readily demonstrable, in the supravital technique, as the predominating reaction, and giving in fixed sections structures which closely simulate the tubercles of the disease.

The criteria for the identification of the living monocytes have been given in a previous publication (11). The essential features are the rosette of small bodies that react to neutral red with a uniform salmon tint and the masses of tiny mitochondria around the rosette and excentric nucleus. The characteristics of the living epithelioid cell stained with neutral red have been described by Cunningham

et al. (12). In our first report on the action of the chemical fractions (3), an epithelioid cell from the blood stream of a rabbit with tuberculosis (Fig. 3) was shown on the same plate with a similar epithelioid cell (Fig. 5) produced chemically by the phospholipin. In the epithelioid cell the rosette is large owing to the enormous numbers of tiny bodies reacting to neutral red as do the fewer bodies of the normal monocyte.

There is some difference of opinion concerning the relationships of the cells of the connective tissues; one group of investigators regard all mononuclear forms as different physiological states of the same strain of cells; another group hold the opinion that they represent different functional strains. The evidence at hand seems to us to give weight to the view that monocytes, epithelioid cells, and the rosette giant cells derived from epithelioid cells, represent a strain of connective tissue cells somewhat different from the macrophages or clasmatocytes. Whether this view be correct or not, the tissue reactions recorded as positive in these experiments embrace a major response of cells answering to the above criteria for monocyte, epithelioid cell, and Langhans' giant cell.

Our first report (3) included the preliminary biological testing of the phosphatide, designated A-3, from the human strain of tubercle bacilli H-37. Four rabbits (R 153, R 158, R 159, and R 160) were given twelve intraperitoneal doses of the phosphatide in amounts averaging 80 mg. per dose, in 10 cc. distilled water. The phosphatide produced massive tubercular tissue in the omentum, under the serosal coat of the intestine, in the mesentery, and under the parietal peritoneal lining. The reaction was predominately one of epithelioid cells and Langhans' giant cells, either diffusely scattered or aggregated into clumps simulating tubercles, as illustrated in Figs. 10, 11, and 12 in an earlier report (3). The animals showed no toxic symptoms; there was no fever and no loss in weight. There was a definite rise in monocytes in the blood, with some qualitative changes suggestive of the transition from monocyte to epithelioid cell, not, however, as marked as in the disease itself. There was in each case a slight fall in red cells and in hemoglobin.

These observations have since been confirmed and extended in a large series of animals.

In the present study, twenty-one normal rabbits (Table II) received the phosphatide A-3 intraperitoneally, uncomplicated by other procedures. Thus, with the four of the preceding report, twenty-five animals have been included in the work. The dose which has been generally used, 80 mg., represents the amount of the phosphatide in 1.6 gm. of bacilli, this substance amounting to 5 per cent by

weight of the dry bacilli. When the phosphatide was first isolated in 1927, it was possible to get a fine dispersion of the particles by shaking the substance in water in a test-tube. Two years after the original isolation, it was found necessary to grind it in a mortar while adding water drop by drop. In this manner a uniform suspension could be obtained in which no flocculi could be seen with a hand lens and from which none settled out on standing.

The original finding of massive tubercular lesions and the fact that the phosphatide is non-toxic *via* the intraperitoneal route have been confirmed. Out of the group of twenty-five rabbits, four became ill, probably independently of the injections, except in so far as there was accidental secondary infection. Two (R 462 and R 321) developed diarrhea; one (R 462) died, but the cause of death could not be determined at autopsy; there was no peritonitis or pneumonia. The other (R 321) was killed and showed no peritonitis, but in one lung there was a patch of atelectasis in which the bronchi were filled with leucocytes and the lymphatics with a coagulum. A third animal (R 463) died of pneumonia, and a fourth (R 376) died of undetermined cause. To the group of twenty-five rabbits, fifteen more may be added, in connection with observations as to the non-toxic nature of the phosphatide. Each of them received twelve intraperitoneal injections of 80 mg. of the phospholipin and was then inoculated with living tubercle bacilli. None of these fifteen animals showed any symptoms from the phosphatide injections.

In the studies with the phosphatide there have been no complications from the presence of a diluting menstruum; the sterile distilled water used was itself inert to the connective tissue cells. With the four exceptions just mentioned, there has been no infection. The phosphatide itself could not be sterilized, but other precautions for sterility were taken, and when there were signs of infection carried in from the skin or from puncture of the intestine by the needle, cultures were taken. Routine cultures were not made. A smooth and glistening serosal lining was taken as evidence of no peritoneal infection.

Twenty-four of the forty animals which received intraperitoneal injections of the phosphatide had complete studies of the blood cells. There was a tendency to an irregular rise of monocytes in the peripheral blood, averaging 20 per cent of the total white blood cells at the highest point, with a range of from 12 to 40 per cent, and total numbers of from 1,250 to 7,500 monocytes per c.mm.

Fifteen of the rabbits showed an average fall in the total red blood cell count of 850,000 per c.mm., and a decrease of 10 per cent in the hemoglobin during the injections (see (3), Charts 4 and 5). To ascertain if this slight anemia should be attributed to the phospha-

tide *per se* or to other factors involved in the experiments, four controls were made.

Rabbit R 709 was given twelve daily intraperitoneal doses of 10 cc. of distilled water; R 730, twelve doses each of 10 cc. normal salt solution; R 664 twelve doses each of 10 cc. normal saline plus 80 mg. of the phosphatide; and R 1016 one dose of 1 gm. of phosphatide in 20 cc. distilled water. The rabbit receiving repeated injections of distilled water (120 cc. total) did not show anemia at any time, whereas the other three animals each developed a definite anemia in the course of the

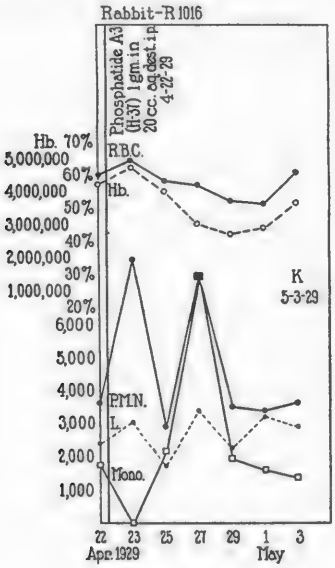

CHART 1

experiment. Chart 1 records the blood changes observed in Rabbit R 1016 over a period of 11 days, following one injection of 1 gm. of phosphatide intraperitoneally in 20 cc. distilled water, an amount of phosphatide equivalent to that usually injected in divided doses over the same period. Not only was there a definite, though transient decrease in red blood cells, but the circulating monocytes, on the 5th day after the injection, reached 7,500.

From these experiments and others in which the intravenous route was employed, it is evident that the phosphatide itself has a tendency to produce an anemia; it is also clear that, as the monocytes are in-

TABLE II

Protocols

The Effect of Intraperitoneal Injections of Phosphatide A-3 from Tubercle Bacilli, H-37, Given in Distilled Water, in Producing Tubercular Tissue in 21 Rabbits

No. of rabbit*	Dose	Results
R 819	1 dose of 80 mg. in 10 cc. aq. dest.	Milk spots of omentum accentuated by phagocytosis of lipoid in large round cells. Marked emigration of leucocytes and dilatation of vessels
R 574 R 820	2 doses of 80 mg.	Milk spots increased in size and number by phagocytic cells. Free leucocytes fewer; many engulfed in clasmatocytes
R 575 R 821	3 doses of 80 mg.	Milk spots more massive and more numerous. Phagocytic cells predominate; leucocytes few and mostly in clasmatocytes. Monocytes and epithelioid cells in active division
R 478	7 doses of 10 mg.	Omentum contains many small groups of epithelioid cells; many dividing
R 482	15 doses of 20 mg.	Omentum, slight general thickening with epithelioid cells
R 462	5 doses of 80 mg.	Developed diarrhea; found dead, cause not determined; no peritonitis. Omentum showed increase in young basophilic mononuclear cells and epithelioids. No tubercle-like structures. No giant cells
R 463	6 doses of 80 mg.	Died of pneumonia. Omentum considerably thickened with diffuse epithelioid cells. Tissues infiltrated with leucocytes. Many abscesses
R 664	11 doses of 80 mg.	Thin central part of omentum showed milk spots of epithelioid cells; rest extensively thickened with tubercular tissue of great variety. Epithelioid cells and epithelioid giant cells
R 319 R 321 R 376 R 380 R 381 R 382 R 383	11 to 14 doses of 80 mg.	Omenta massively thickened with tubercular tissue. Peritoneum extensively involved. Omenta of R 376, R 380, and R 381 used by Dr. Anderson for recovery of a phosphatide by chemical analysis. Omenta of R 319 and R 382 used by Drs. Richardson, Lobell, and Schorr for respiratory tests. R 321 developed diarrhea and was killed; tissues showed marked necrosis. R 376 died, cause undetermined

* These are serial numbers of the work of the department covering a term of years.

TABLE II—*Concluded*

No. of rabbit	Dose	Results
R 1016	1 dose of 1 gm. in 20 cc. aq. dest.	Central area of omentum was thin but had large milk spots of epithelioid cells. Rest of omentum massively thickened with tubercular tissue; typical epithelioid cells and epithelioid giant cells with fine neutral red bodies predominating
R 368	13 doses of 80 mg.	Killed 1 mo. later. Excellent state of nutrition; gain in weight from 2,300 to 2,750; much yellow adipose tissue; adhesions opposite place of injections. Omentum adherent; considerably thickened with scattered epithelioid cells and small tubercle-like masses. Richly vascularized
R 377	" "	Killed 3 mos. later. Excellent condition; gain in weight 1,800 to 2,475. Omentum much less thickened but had more adipose tissue than usual. No scattered epithelioids but several compact nodules with a few epithelioid giant cells
R 379	" "	Killed 5 mos. later; gain in weight from 1,840 to 2,550. Adhesions opposite place of injection. Omentum moderately thickened with fibrous tissue. Only two nodules of epithelioid cells large enough to see in the gross. No diffuse epithelioid cells but a few small clumps

creased in the tissues, irregular showers may appear in the peripheral blood. Further analyses of the effects of the intravenous phosphatide on the bone marrow are being made and will be reported later.

The injection of living or dead tubercle bacilli is followed in the first 24 hours by an emigration of leucocytes from the vessels into the tissues and their subsequent phagocytosis by clasmatocytes. Within 4 days epithelioid cells appear, followed by the development of tubercles (Kageyama, 13, and Lewis and Sanderson, 14). The same acute response, an emigration of leucocytes and their destruction by clasmatocytes, follows the intraperitoneal injection of every substance isolated from the bacillus. The different substances vary however considerably as to whether repeated injections cause a fresh emigration of the leucocytes or not (Table II).

Of all the lipoidal substances it is the phosphatide, not the so-called waxes, that produces the most massive tubercular tissue. The reac-

tion to the injection of the phosphatide A-3 following one, two, and three doses is, however, complex and does not consist in the immediate formation of epithelioid cells either diffuse or in tubercles. After from seven to fourteen doses the formation of massive tubercular tissue is constantly found.

24 hours after the first dose (Rabbit R 819), the most striking change in the peritoneal cavity was in the omentum and consisted in the accentuation of the milk spots (protocols in Table II). This was due in part to the emigration of large numbers of neutrophilic leucocytes into the milk spots, but more to the fact that all the cells of the milk spots had rounded up and became highly phagocytic both to the lipoid and to leucocytes. Since the milk spots of the normal omentum in the rabbit are made up of monocytes, young clasmatocytes, and still more primitive cells, all of these types must be involved in the original phagocytosis of the phosphatide.

After the second dose (Rabbits R 820 and R 574), the milk spots were denser with phagocytic cells, but the free leucocytes were less in number because those called out early had been largely phagocytized and the second injections did not produce as much irritation as the first. Following the third dose (Rabbits R 821 and R 575), only a few leucocytes remained either free or in clasmatocytes, and the further injections of the phosphatide did not call out more leucocytes from the vessels.

At first the process was limited to the original milk spots; but by the third dose new milk spots were forming. In the supravital preparations the dense milk spots were made up of large round cells of nearly uniform type heavily loaded with large vacuoles which stained a uniform red color in neutral red. In fixed sections these cells appear highly vacuolated (Plate 1, Figs. 1–3, Rabbit R 821), the lipoidal substances having dissolved out in the dehydration and embedding. Stained for fat, these cells were negative to Sudan III; with Scharlach R most of the material was negative, but along the edges of the large, unstained globules were great numbers of tiny droplets which stained with the characteristic reaction of a fat. The phospholipin itself did not stain either with Sudan III or with the Scharlach R, so that if this intracellular material which gives the fat reaction came from the phosphatide, it must have been the product of some change within the cells.

On Plate 1, Fig. 1, one of the vacuolated cells (upper left corner) contains a leucocyte partly digested, and in Fig. 2 there are two such cells, each containing a leucocyte with the nucleus still recognizable, and a third, near the center, showing only débris. This reaction after three doses is minimal, and it will be noted that there is only one free leucocyte (Fig. 2) in the three figures.

The milk spots as studied in the fresh omentum and in sections had a few smaller cells, well shown in all three figures, and identified as monocytes and epithelioid cells. Two cells are clearly shown along the left border in Fig. 1, one of them with

the dense cytoplasm and excentric nucleus characteristic of a small epithelioid cell. A small group of young mononuclear cells is in the center of Fig. 3, two of them in division. This material showed much cell division, most frequently in the typical monocytes; the highly vacuolated cells however showed an occasional nuclear figure, while the endothelium of the small vessels and the adventitial cells were dividing freely. In this tissue on the 4th day after the first dose, the vacuolated cells predominated, while monocytes and typical epithelioid cells were in minimal numbers; but later the reverse was the case, the maturation of new monocytes and their transformation into epithelioid cells becoming the dominant reaction.

During the 2nd week of the reaction in the omentum to repeated injections of lipoids, the adipose tissue became gradually replaced by the new tubercular tissue and during this process fat cells appeared vacuolated in sections, owing to the breaking up of the fat into smaller globules. In the shrinking fat cells the droplets readily reacted to Sudan III, and thus these cells could be discriminated from the cells of the early reaction to the phosphatide in which most of the material was negative to fat stains.

All the animals in Table II which received from seven to twelve doses of the phosphatide exhibited a formation of tubercular tissue, the amount of which was in a general way proportional to the dose.

Rabbit R 478 had seven daily intraperitoneal doses of 10 mg. of the phospholipin in 2 cc. of distilled water and the omentum was studied 24 hours after the last dose. In this tissue, small clumps of epithelioid cells were found (Plate 2, Fig. 5). The total amount of the phosphatide given, 70 mg., was less than the average single dose, 80 mg. In this rabbit the omentum had accentuated milk spots but there was not enough of the new tissue to cause in the interspaces any change detectable with the unaided eye; nor was there any replacement of the fat by the specific tubercular tissue, that is, there was no thickening of the membrane. This condition in which only the milk spots are involved represents the first stage in the reaction in the omentum. Supravitally the milk spots themselves consisted predominantly of epithelioid cells. Some of them had two nuclei, but no larger giant cells were seen. The sections also show that the milk spots are clumps of epithelioids; in Fig. 5 is a clump of about twenty typical epithelioid cells, one of which is in division. The cells show the dense cytoplasm and the excentric nuclei characteristic of epithelioid cells of the actual infection. Note the absence of leucocytes both in this figure, and in Fig. 6.

In Fig. 6 is shown a similar small tubercle-like structure taken from the wall of the cecum just under the serosal coat in a rabbit (R 319) which had twelve intraperitoneal doses of 80 mg. of the phosphatide. It will be observed that these epithelioid cells have a much less dense cytoplasm than those of Fig. 5; in fact, except for a central core the entire cytoplasm appears foamy. In the supravital

technique, these cells had larger bodies reacting to neutral red than those in the typical epithelioid cell. This difference in size of neutral red bodies can be seen in the photographs from living cells from Rabbit R 664 (Plate 1, Fig. 4, and Plate 3, Figs. 7 and 8). This rabbit received eleven doses of 80 mg. of a phosphatide sample 2 years old. The reaction was typical but not as massive as with the animals experimented on earlier with the same dosage. In the omentum of this animal (R 664) there was in the gross a marked accentuation of the milk spots, shown in Fig. 4, but not enough change in the interspaces to be detected with the unaided eye. Fig. 4 is not from a section but from a spread of the omentum supravitally stained with neutral red and photographed while the cells were still living. The milk spots showed an almost uniform reaction; the cells in the center of the photograph are paler, merely because they were beginning to fade. This is perhaps as pure a reaction toward one type of cell as can be obtained in the tissues of the animal; it is, so to speak, a culture of epithelioid cells in the omentum. Fig. 7 shows the edge of one of the milk spots in the same omentum at a higher magnification; there is one giant cell of the rosette type. This giant cell and the epithelioid cells near it show the bodies which react to neutral red, and photograph dark, as larger and more irregular in size than the neutral red bodies in the two cells of Fig. 8, taken from an interspace of the same omentum. In the latter figure the bodies are finer and more densely packed. In this animal the vast majority of the cells were of the type with the coarser neutral red bodies, and in fixed sections the majority of the epithelioid cells had a foamy cytoplasm intermediate in type between the cells of Fig. 5 and Fig. 6; the cytoplasm was dense with a high dry lens but appeared foamy with oil immersion lenses. The sections of this material (Rabbit R 664) showed much tubercular granulation tissue, many epithelioid giant cells, and many tubercle-like structures.

To test whether time was a factor in regard to these two types of epithelioid cells, the one with the foamy cytoplasm and the other with the dense cytoplasm, a rabbit, R 1016, was given in one dose the total amount of the phosphatide usually given in twelve divided doses, namely, 1 gm. in 20 cc. distilled water intraperitoneally. It was killed 12 days later. The changes in the blood have already been referred to (Chart 1). The middle portion of the omentum showed large milk spots with no thickening of the interspaces; and the rest of the omentum was dense with nodules of pink tissue markedly resistant on cutting. There was also some of the specific tissue, that is, some epithelioid cells, in the thoracic lymph nodes, along the internal mammary veins, and in the ventral pericardium. Supravitally the clumps of new cells in the omentum were found to be predominately epithelioid cells with typical fine neutral red bodies, but here and there was one with the coarse granules corresponding to an occasional epithelioid cell with foamy cytoplasm (like the cells of Fig. 6 in the sections). This was the same material that gave a predominance of the epithelioid cells with coarse granules when given in divided doses. There were also many typical epithelioid giant cells. The experiment suggests that the epithelioid cells with the coarse granules

become the cells with the fine neutral red bodies if sufficient time is allowed; or that if the cell contains some substance which it has phagocytized, it has the power of breaking this substance into finer and finer particles.

The second stage of the extreme reaction from twelve doses of 80 mg. of the phosphatide given intraperitoneally, which was described in the original report upon four rabbits, has been studied in seven others. The tissues from these animals were examined supravitally to confirm the presence of a massive development of the tubercular tissue, and then the omenta were used either for the chemical recovery of the phospholipin by Dr. R. J. Anderson, or the tissues were used by Dr. H. B. Richardson, Dr. R. O. Lobell, and Dr. E. Schorr of Cornell University, New York, for studies on the respiratory rate of the cells.

With this dosage of 80 mg., the omentum became massive with tubercular tissue (Figs. 9–13) within 12 to 14 days, being transformed into a dense membrane with many irregular nodules at least 2 to 3 cm. thick. The reaction was not one to an acute infection; it was not a peritonitis of bacterial origin; there was no fibrin; there was no tendency to adhesions; except when there was an accidental pyogenic infection, there was no infiltration with leucocytes after the repeated doses, except in relation to the nodules of caseation; the serosal lining was intact, but beneath the serosa were extensive masses of the new tissue. In this massive reaction not only the thin part of the omentum showing milk spots but the denser areas normally filled with fat cells became involved; these cells disappeared almost entirely and their place was taken by the tubercular masses. Beside the effect on the omentum, there was a widespread subserosal reaction in the peritoneal cavity, as shown by extensive tubercular tissue in the mesentery, the outer coat of the cecum, the capsule of the spleen, the mesentery between liver and stomach, the serosa of the liver, the diaphragm, the parietal peritoneum, and the connective tissue around the bladder and the reproductive organs. These lesions were scattered over so extensive an area that it was not possible to estimate the actual amount of new tissue formed in the 2 weeks of the experiment, but judging from the omentum itself the entire mass was probably comparable in size to a hen's egg, a rate of growth of the new tissue resembling that of a rapidly growing neoplasm.

The proliferation induced by the phosphatide was of epithelioid cells and epithelioid giant cells as the predominating fundamental units, different combinations of the two giving great variety to the reaction. Of all the lipoidal substances, the phospholipin yielded not only the most massive lesions but the greatest variety in different combinations of epithelioid cells and giant cells.

On Plate 3, Fig. 9 (R 158) is shown one of the densest lesions. It is a small part of an extensive, solid mass of epithelioid cells with only a few giant cells; two are shown, one along the upper border and the other near the upper left corner. At the bottom of the figure is a large area of lymphocytes around blood vessels. Between the epithelioid cells are short lines of lymphocytes. The tissue shows practically no free leucocytes. Except at one end the tissue is poor in vessels.

A large mass of tubercular tissue, at a low magnification, is shown in Fig. 10, Rabbit R 153. In this tissue there are definite small clumps of epithelioid cells simulating tubercles; the main mass contains a large percentage of epithelioid giant cells and there are two masses of lymphocytes. Fig. 11, Plate 4, from the omentum of the same animal shows a combination of tubercular granulation tissue and small clumps of epithelioid giant cells. In the upper part is a mass of giant cells infiltrated with lymphocytes in tissue quite richly vascularized, while below is a zone of scattered epithelioid cells. These are quite clearly the typical form with dense cytoplasm. There are no free leucocytes. A lesion of tubercular granulation tissue, with almost no tendency to clumping, is shown in Fig. 12 (R 153). A lesion made wholly of epithelioid giant cells is shown in Fig. 13 from a small lymph node along the internal mammary vein of Rabbit R 319, which had twelve intraperitoneal injections of the phospholipin. At autopsy this lymph node looked like a mass of the specific tissue and was infiltrated with giant cells which in certain areas had almost replaced the follicles and the lymph cords. Such cells were not found in the sinuses. There occurred but little transportation of the phosphatide following these intraperitoneal injections; in one animal (R 158) the specific tissue was found in the mesenteric lymph node; in another (R 319) in a thoracic node; in a third (R 1016) also in the thoracic nodes and the ventral pericardium; otherwise, the reaction has been restricted to the peritoneum.

The study of the chemically produced tubercular tissue offers a chance to study caseation in a simplified form. There have been differences of opinion as to whether leucocytes in caseous material are a response to necrosis or represent a secondary infection. Medlar (15) has recently emphasized the invasion of tuberculous tissue with leucocytes in the absence of secondary infection, an occurrence which is readily explained by the reactions to the chemical fractions from the bacillus. In the response to the various fractions, the phosphatide has been found to possess only a limited power to call out leucocytes into the tissues, leucocytes responding actively only to the first dose. With other substances, however, the undiluted fatty acids and the polysaccharides, for example, each succeeding dose is as irritating as the first. From these observations, it might be inferred that during the disintegration of tubercle bacilli in the tissues, the number of free

leucocytes might vary greatly in accordance with the extremely complex chemical process of disintegration of bacilli and tissues, but that the presence of some free leucocytes might be expected. Auclair (16) reported caseation in rabbits and guinea pigs, produced by lipoids from the tubercle bacillus, but Ray and Shipman (6), in the study of ether-soluble lipoids, considered the areas of necrosis to be only ordinary pus.

In the tubercular tissue produced by the phosphatide, a certain amount of necrosis develops which cannot be discriminated histologically from caseation.

After twelve doses there have always been a few small, yellow nodules to be seen in the gross filled with a cheesy débris different in appearance from the thick, white pus of rabbits. These nodules have had a center of necrotic tissue usually surrounded by a border of granulation tissue. The process, however, is much better studied in tiny microscopic nodules where the cells show only early signs of damage instead of disintegration. The sections of tubercular tissue procured after twelve doses of the phosphatide, consisted mostly of masses of epithelioid cells, with hardly a free leucocyte. Here and there were small areas of damaged epithelioid cells surrounded by free leucocytes. The absence of any signs of a general inflammatory reaction in the tissue suggests that the death of epithelioid cells is the initial process, but it is probable that the invasion with leucocytes takes place so quickly as to make the two processes almost synchronous. Nodules can be found so small that the outline of each damaged epithelioid cell can still be seen. There is almost no phagocytosis of leucocytes in such foci, but occasionally a single leucocyte is found engulfed. Fig. 14, depicting a small caseous focus from a lymph node of Rabbit R 319, shows the next stage in the process. The epithelioid cells of the border are markedly vacuolated with only an occasional leucocyte among them; the center is a mass of débris in which nuclei of epithelioid cells and leucocytes can be made out. These observations lead to the view that simple caseation is a process in which dead epithelioid cells are invaded and changed by leucocytes.

A process entirely different from caseation has been shown by Doan and Sabin (17) to take place in the regression of tubercles in the bone marrow of rabbits with bovine tuberculosis. The bone marrow always became involved with extensive tubercles after massive (1 to 2 mg.) intravenous injections of the bovine strain, but the lesions did not persist there. The individual epithelioid cells disintegrated and the débris was taken up by clasmatocytes. In Fig. 5 of the paper referred to, dying epithelioid cells can be seen in an area of developing red blood cells, without either myelocytes or leucocytes in the neighborhood.

The fate of the tubercular tissue was followed in the last three rabbits of Table II. From the work of Prudden and Hodenpyl (18, 19), it has been known that the tissue produced by dead tubercle bacilli is gradually resorbed.

Rabbits R 368, R 377, and R 379 all received 13 intraperitoneal injections of 80 mg. of phosphatide, a dosage which was proved at autopsy to have produced massive tubercular tissue in the peritoneum of other rabbits of the same lot. Rabbit R 368 was killed 1 month, R 377, 3 months, and R 379, 5 months after the last injection. All three remained in excellent condition throughout the period of observation, gaining in weight. All had shown monocytes as many as 20 per cent early in the experiment. During the phase of resorption no anemia developed. After 1 month (R 368), the omentum was still greatly thickened and there were many of the pink nodules of tubercular tissue. The most striking point was the marked vascular organization of the tissue; there were still scattered epithelioid cells. There were adhesions in this animal between the omentum and the intestine and between the body wall and the intestine at the point where the injections had been made, so that there may have been some infection. The amount of tubercular tissue remaining at 3 months was intermediate in amount between that at 1 and 5 months. In R 377, the omentum was still thickened, but much of the bulk was due to the return of fat. Supravitally there were clumps of epithelioid and giant cells, and an occasional deep pink nodule was found. It consisted almost entirely of epithelioid cells. In R 379 the omentum after 5 months showed an increase in fibrous tissue, but there was no fibrous capsule around the very few remaining tubercular nodules. In this tissue, adventitial cells, loaded with cellular débris, marked out the blood vessels as well as an artificial injection would have done. These three animals thus showed different stages in the disappearance of the chemically produced tubercular tissue, the diffuse reaction disappearing first. After 5 months, only one or two nodules of the specific tissue were still present in the omentum.

From our observations, then, it appears that disintegrating epithelioid cells may be rendered caseous by the infiltration of leucocytes or may be phagocytized by clasmatocytes without caseation. It is probable that the regression of the chemically produced tubercular tissue is accomplished largely by the latter process, as is that in the bone marrow in the disease itself, since caseation is found in this organ only occasionally.

SUMMARY

The immediate response of the tissues to the injection of the phosphatide A-3 indicates that the material is phagocytized by connective

tissue cells which then appear highly vacuolated. Epithelioid cells appear after three doses, perhaps only after the phospholipin has been to some extent broken down by cells. The increase in tissue occurs largely through the maturation of new monocytes and their transformation into epithelioid cells. During the 2nd week, following repeated daily injections, or after one massive dose, there is an extensive production of epithelioid cells and giant cells, forming typical tubercular tissue in the peritoneum. This tissue shows great variations, consisting either of diffuse granulation tissue, multiple small nodules, or massive aggregations of epithelioid cells. Giant cells are mainly of the simple epithelioid type; the tissues may show only an occasional giant cell or they may predominate or be indeed the only cell present.

Some of the varieties in the types of tubercular tissue produced have been illustrated in the photographs; these figures do not, however, cover the whole range of combinations. The tissue has a greater tendency to vascularization than in the reaction of the actual disease. Some of the small clumps of epithelioid cells are non-vascular; others of the same size possess vessels. The tissue undergoes complete resorption if given sufficient time, in small part by caseation, but to a greater extent probably through the death of the epithelioid cells and the elimination of the débris by the phagocytic action of clasmatocytes.

The general discussion of the effects of the lipoids and of the subfractions from them is given on pages 60 to 69.

REACTION TO THE FATTY ACIDS, THE WAXES, AND THE GLYCERIDES

As shown in Table I (p. 7), the phosphatide was broken down by Dr. Anderson (9) into six simpler substances: oleic acid, palmitic acid, glycerophosphoric acid, glucose, a sugar acid, and a hitherto unknown liquid saturated fatty acid, designated fatty acid I. The first three substances failed to produce tubercular tissue; all of them, however, were irritating, and caused an emigration from the blood vessels of neutrophilic leucocytes, which subsequently suffered phagocytosis by clasmatocytes, while the administration of the palmitic acid was followed by a considerable reaction of normal monocytes. The glucose and the sugar acid have not been tested.

Effects of the Fatty Acid I

Fatty acid I is a clear, colorless, heavy oil. It is thermostable and was sterilized by heat before being used for the injections. Since Dr. Anderson has given us a part of the original material for biological testing, he has not yet had enough of this acid for complete analysis; he considers, however, that it is a mixture of two or more fatty acids, which possibly differ in their optical activity.

The fatty acid I, in contrast to the other phosphatide subfractions, gave an extensive formation of tubercular tissue (Plate 5), much of it in the massive reaction characteristic of the phosphatide, but more in the form of tubercle-like aggregations of epithelioid cells, with some epithelioid giant cells, the tissue closely resembling that of the actual infection. It thus appears that in the phosphatide the active principle for the production of typical tubercular tissue is carried by this fatty acid. These two substances, the phosphatide and its saturated fatty acid, are far more potent in producing tubercular tissue than the other principal fractions of the lipoids, namely, the so-called waxes and glycerides, and their partitions.

There is no accurate way of determining the total amount of tubercular tissue produced by any fraction, spread out as it is over a wide surface underneath the peritoneal serosa and in the omentum. For the purposes of comparison we may designate the amount produced by the phosphatide ++++; the amount of tissue resulting from its fatty acid is almost, if not quite, as great, and might be indicated by the same symbol.

Fifteen rabbits have been tested with this acid intraperitoneally, as is shown in Table III.

The dose given, 0.1 cc. (16 mg.), was the amount of the fatty acid contained in 80 mg. of the phosphatide. The fatty acid was first injected undiluted into the peritoneal cavity; seven animals were used in this experiment. Rabbit R 397 received one dose of 16 mg. intraperitoneally and was studied 24 hours later. The abdominal wall showed some hyperemia at the site of the injection, as did the underlying intestine. The omentum was stimulated, with accentuated milk spots, and showed the same pink color as with the phosphatide. In the supravital examination the most striking point was the great number of monocytes; many of these monocytes were young forms and in some areas they were by actual count the predominating cell. In the preparations there could be no confusion between

TABLE III

Protocols

Effects of Intraperitoneal Injections of Fatty Acid I, from Tubercle Bacillus, H-37, in 15 Rabbits

No. of rabbit	Dose	Diluting menstruum	Results
R 397	1 of 16 mg.	None	Accentuated milk spots; dilated vessels; neutrophilic leucocytes; young monocytes; clasmatocytes not active
R 399	2 of 16 mg.	None	Omentum more stimulated than in R 397; leucocytes being phagocytized by clasmatocytes. Serosal cells irritated. Few epithelioid cells
R 445			Marked hyperemia of body wall, and intestine adherent; may have been small perforation; some monocytes; marked stimulation of clasmatocytes and serosal cells
R 401	3 of 16 mg.	None	Omentum increased milk spots; in some areas abscesses and zones of necrosis. Elsewhere relatively little infiltration of neutrophilic leucocytes but great increase in monocytes and epithelioid cells. Border of mesenteric lymph node of tubercular tissue
R 446			Cecum and colon covered with exudate and adherent. Supravitally marked maturation of monocytes but signs of irritation predominate
R 619	10 of 16 mg. every 24 hrs.	None	Dense nodular adhesions of cecum and colon to body wall; surface of small intestine and rest of body wall smooth and glistening. Extensive cell division in mononuclear forms and some epithelioid cells. Serosal cells of omentum filled with refractile bodies
R 620	5 of 16 mg. every 48 hrs.		Dense adhesions between cecum and body wall and other loops of intestine with white nodules (pus); serosal cells of omentum filled with refractile bodies; clasmatocytes loaded with cellular débris; marked increase in monocytes; few epithelioid cells and giant cells
R 334	15 of 20 mg.	5 cc. olive oil	All abdominal organs matted together with adhesions. Cultures negative. Supravitally no normal connective tissue cells. Marked stimulation of clasmatocytes. Remarkable for lymphocytes and for giant cells; some epithelioid giant cells and some foreign body types, 20 to 50 per field. Extreme variation in tissues

TABLE III—*Concluded*

No. of rabbit	Dose	Diluting menstruum	Results
R 335			All abdominal organs matted together with adhesions; cultures negative. Remarkable for lymphocytes and for giant cells, predominantly very large foreign body types with many nuclei. Some epithelioid cells. Tissue fibrous and not densely cellular
R 336	15 of 20 mg.	5 cc. olive oil	Died, peritonitis, colon bacillus. Moderate number of giant cells predominantly foreign body types. Tissues very cellular; neutrophilic leucocytes; clasmatocytes; epithelioid cells
R 337			No peritonitis; cultures negative. Omentum and mesentery thickened and looked like tissue from the phosphatide. Remarkable for lymphocytes and for giant cells, predominantly of foreign body type. Some epithelioid cells
R 510	3 of 16 mg.	0.5 cc. mineral oil	Peritonitis; wall of cecum, omentum, capsule of liver and spleen showed white nodules of pus. In the tissues free neutrophilic leucocytes predominated; a few were in clasmatocytes. There had been a considerable maturation of monocytes, and supravitally a few epithelioid cells were identified
R 502			Adhesions between cecum and body wall. Median lobe of liver and spleen had thickened capsule. Omentum massively thickened, pink, nodular. Epithelioid cells, mainly in tubercle-like structures with lymphocytes. One massive, extensive lesion. Few epithelioid giant cells. Neutrophilic leucocytes in small foci. Extensive replacement of the adipose tissue. No free fluid
R 613	12 of 16 mg.	0.5 cc. mineral oil	Entire peritoneum involved with pink, nodular tissue. Omentum massively thickened. Epithelioid cells, largely in tubercle-like structures with lymphocytes, but more tubercular granulation tissue than in R 502. Few epithelioid giant cells. Partial replacement of adipose tissue. No free fluid
R 614			Entire peritoneum involved with pink, nodular tissue. Omentum, many small tubercle-like structures with and without lymphocytes. Reaction most massive around reproductive organs. Few epithelioid giant cells; neutrophilic leucocytes in small foci

clasmatocytes and monocytes, since all of the former were large, mature, branched, unstimulated cells, while the monocytes were all smaller, round cells with typical rosettes. The mesentery also showed young monocytes and mature unstimulated clasmatocytes. There were extensive infiltrations with neutrophilic leucocytes, which in fixed sections appeared in the form of multiple small abscesses as the major reaction. The sections showed small clumps of basophilic mononuclear cells, but this reaction would probably not have been recognized without the records of the supravital studies.

Rabbit R 399, which received two injections, had the same accentuation of milk spots as the preceding animal, except that there were a few white nodules of pus large enough to be seen in the gross. Supravitally interesting changes were seen. There was the same marked irritation shown by the leucocytes; the clasmatocytes, however, instead of being unstimulated, were now active in engulfing the damaged leucocytes, and among the monocytic strain there were some epithelioid cells. The bone marrow showed a shift toward B-myelocytes, indicating the extent of the drain of the leucocytes from the blood stream.

Rabbit R 445 also received two doses. The intestine was adherent to the body wall and may have been perforated. Supravitally there was observed a marked stimulation both of serosal cells, as indicated by an increase of refractile bodies in them, and of clasmatocytes as shown by increased phagocytosis. There were some monocytes and a few epithelioid cells, but the most striking reaction was the acute peritonitis.

Two rabbits, R 401 and R 446, were given three injections. In R 446, signs of irritation predominated, for the cecum and colon were covered with exudate and had fresh adhesions. In the omentum there had been an active maturation of monocytes, but this reaction was not as striking as the infiltration with leucocytes and the activity of the clasmatocytes. In R 401, on the other hand, there was not much general irritation. In the omentum there were some abscesses and a few zones of necrosis, containing dead mononuclear cells staining heavily in eosin, and many granulocytes, both neutrophilic and eosinophilic; but elsewhere there was but slight infiltration with leucocytes, and the reaction, both as seen supravitally and in sections, was predominantly monocytic, with many epithelioid cells. Some of these had more than one nucleus showing a tendency toward giant cell formation, but they were not much larger than epithelioid cells. In sections the monocytic and epithelioid reaction was sufficiently definite and striking to be noted, even if the supravital studies had not called attention to it. The tissue did not look like the tubercular granulation tissue, nor like the tubercle-like masses illustrated for the phosphatide, for there had been no great thickening of the omentum, no great increase in blood vessels; but in a relatively normal omentum there was this marked increase in the one strain of cells. Very few plasma cells were found, but there were many clumps of deeply basophilic mononuclear cells, as well as typical monocytes and epithelioids, some of them in division, both scattered and in small clumps of 8 to 10 cells.

The acute lesions produced in experiments with from one to three doses demonstrated the extremely irritating character of the undiluted fatty acid. Except in one rabbit (R 445) in which the wall of the intestine may have been punctured by the injection needle, it was judged that the irritation was due to the acid itself and not to injury or infection. There was considerable evidence of a stimulus toward monocytes, especially plain in Rabbit R 401. There were none of the large vacuolated cells that characterized the early reaction to the phosphatide, which were regarded as probably indicating the phagocytosis of the phosphatide, but rather a tendency was observed to a direct maturation of monocytes from more primitive forms.

The remaining animals receiving fatty acid I were given from 3 to 15 repeated doses, and, with one exception (R 502), studies of the peripheral blood were made. The changes in the blood cells (Table IV) were not marked. All the animals showed a slight drop in the number of red blood cells and in hemoglobin, but the bone marrow revealed no obvious changes. In two animals, R 613 and R 620, there was a moderate leucocytosis due to a rise in neutrophilic leucocytes; the monocytes tended to increase and the lymphocytes to decrease in the majority of the rabbits, the most striking changes being noted in R 620, R 334, and R 337.

Two of the rabbits (R 619 and R 620) were given 10 and 5 intraperitoneal injections of 16 mg. of undiluted fatty acid I. In both the irritation was extreme, the intestine, especially the cecum, being bound to the body wall. The intestine was covered with fibrin and exudate; the omentum and mesentery were extensively involved with a reaction of extreme complexity. There was an increase in fibrous tissue, most marked in R 619, making many adhesions; there were many areas of necrosis in some of which were great masses of damaged cells, looking like epithelioids (R 620); in the border of these areas, clasmatocytes, loaded with cellular débris, were present in such numbers as to make them the predominating cell of the reaction. There were masses of granulation tissue, markedly cellular and richly vascularized. Scattered diffusely in this tissue were epithelioid cells and epithelioid giant cells, either singly or in small clumps, but not in sufficient numbers to make typical tubercular granulation tissue. This was interesting in view of the fact that this same fatty acid, when subsequently diluted with a bland oil, gave a reaction so closely like tuberculosis that it could be discriminated only by the entire absence of tubercle bacilli (Plate 5). With the undiluted acid, however, in repeated doses, the extreme irritative reaction appeared to minimize and certainly obscured the reaction toward tubercular tissue. In the omentum the fat cells were in process of changing, which also added to the confusion of the picture. In Rabbit R 620 there were small nodules of epithelioid cells in a Peyer's patch; in neither animal were the mesenteric nodes involved.

Olive oil, 5 cc. per dose, was first tried as a menstruum to dilute the fatty acid

TABLE IV

Blood Counts in Eight Rabbits Receiving 10–14 Intraperitoneal Injections of Fatty Acid I

No. of rabbit	Original white blood cell count and neutrophilic leucocytes (per c.mm.)	Highest white blood cell count and neutrophilic leucocytes (per c.mm.)	Original red blood cells (per c.mm.) and hemoglobin	Final red blood cells (per c.mm.) and hemoglobin	Number of (per c.mm.)			
					Monocytes		Lymphocytes	
					Originally	Highest	Originally	Highest*
R 334	12,300 3,936	13,300 9,310	5,930,000 61%	4,950,000 49%	1,476	1,862	6,150	1,596
R 335	10,300 4,841	10,900 8,502	6,280,000 58%	5,240,000 53%	206	1,599	4,017	6,150
R 336	10,700 4,387	14,200 6,248	5,460,000 60%	4,880,000 50%	1,177	1,344	4,173	2,184
R 337	11,800 4,956	11,800 9,086	6,340,000 72%	5,740,000 60%	1,584	1,416	3,776	944
R 613	6,850 2,329	22,300 17,171	5,190,000 58%	4,690,000 55%	685	1,666	2,877	1,368
R 614	7,600 2,736	8,700 2,523	5,570,000 69%	4,940,000 69%	684	1,914	3,572	2,784
R 619	6,500 2,950	10,450 5,956	5,180,000 73%	4,360,000 67%	455	1,202	2,340	2,682
R 620	5,850 2,808	20,100 10,653	4,990,000 73%	4,300,000 Lost	234	5,226	2,691	2,211

* At time of highest monocytes.

(20 mg.) in four animals, R 334, R 335, R 336, R 337, while at the same time five animals as controls were given the same number of injections of the olive oil alone. The irritation produced with the combination was much more marked than with the undiluted fatty acid, and the controls demonstrated the very marked tendency of olive oil to produce foreign body giant cells. In three rabbits, R 334, R 335, and R 336, all the abdominal viscera were matted together with dense adhesions; one, R 336, died and the colon bacillus was recovered as a probable participant in the general peritonitis. All the tissues were remarkable for the great numbers of giant cells, of both types, but with the large foreign body forms predominating; fifty per low power field were not uncommon numbers, and many of them were vacuolated. Next to the giant cells, multiple small masses of lymphocytes were the most striking part of the reaction, especially in R 334, R 335, and R 337. Lymphocytes were not a feature of the controls with olive oil alone. The tissues were markedly uneven; there was much granulation tissue, and in some areas new connective tissue cells were closely packed together. In other places the tissue was open. In R 334, supravitally studied, not a single normal connective tissue cell was seen. The clasmatocytes were stimulated; there were some nests of epithelioid cells, most marked in R 337, and all the tissues showed epithelioid giant cells. Nevertheless, the sections did not simulate the lesions of tuberculosis. Yet the group, as a whole, tended to show more epithelioid cells and Langhans' giant cells than the controls with olive oil alone.

The results with repeated doses of the undiluted fatty acid and with repeated doses of the fatty acid mixed with olive oil were not as suggestive of tuberculous tissue as those with the few doses of the undiluted acid, in which a maturation of monocytes had been so marked. It was clear that the undiluted acid was too irritating for repeated doses, and that with olive oil the extreme reaction of the menstruum itself toward foreign body giant cells obscured the tendency toward monocytes. Therefore a series of experiments were undertaken to find if possible a relatively inert oil, with the result that a pure mineral oil (Nujol) was eventually selected for use. It proved to be an excellent menstruum. It was practically inert in one, two, and three doses; and with twelve injections, its effects were so much simpler and so much less extensive than with other menstrua tried, and especially the reaction toward epithelioid cells was so very slight that the oil has been used during the past year as a general diluting medium for the fatty acids studied. It has also been used as a solvent for the waxes and their derivatives.

In Fig. 19 on Plate 6 there is, however, shown a film of omentum, supravitally stained with neutral red and photographed while the cells were living, to illustrate the relative uniformity of the reaction to twelve intraperitoneal injections of mineral oil (Rabbit R 661). There had been an increase in number of fibroblasts and clasmatocytes. This film is to be compared with a similar film of omentum photographed while the cells were alive, after eleven doses of the phosphatide (Plate 1, Fig. 4). In sections there were a few giant cells, predominately of the foreign body type, and an occasional epithelioid cell.

One rabbit, R 510, given three injections of the fatty acid in mineral oil, showed a general peritonitis. In spite of the great infiltration of the tissues with leucocytes and zones of necrosis, the omentum showed areas filled with monocytes; in the fresh tissues a few typical epithelioid cells and giant cells derived from them were seen, and they can be identified in sections.

Three rabbits (R 502, R 613, and R 614) were given twelve intraperitoneal injections each of 16 mg. of fatty acid I in 0.5 cc. mineral oil. Rabbit R 513, with mineral oil alone, was the specific control for R 502, and Rabbits R 621 and R 622 for the others. The reaction in these rabbits and their controls, as seen in the gross specimens at autopsy, made the most striking contrast; in each case the reaction in the control rabbit with mineral oil alone was slight, as compared with the massive production of new tissue in the rabbits with the fatty acid.

Instead of an excess of free oil in the peritoneal cavity, which is always present after administration of the mineral oil alone in these doses, the rabbits with the fatty acid plus mineral oil had a peritoneum so dry that no peritoneal fluid could be obtained for the study of the free cells. All three rabbits showed an extensive development of tubercular tissue. There was an extensive involvement of the peritoneum; the omentum, the entire wall of the cecum, the diaphragm, the capsule of liver and spleen, and the connective tissue around the reproductive organs were massively involved with new tissue, pink in color like that from the phosphatide. In some zones this new tissue was in thick, dense layers; in others it was in nodules. There was never as complete an absence of neutrophilic leucocytes in the tissues as after repeated injections of the phosphatide; some leucocytes, both scattered and in small foci, were present in all the sections, but they formed a minor part of the reaction in comparison to the epithelioid cells.

The tissue reaction of Rabbit R 502 is represented by a quite characteristic tubercle-like focus taken from the omentum (Plate 5, Fig. 15). It is densely surrounded by lymphocytes; there were no giant cells in this area but a few of both types were found in the sections. In the wall of the cecum of this animal were extensive layers of epithelioid cells as large and as dense as in Plate 3, Fig. 9, from the phosphatide. In some of the blocks fixed from the omentum and from the capsule of the spleen there were large abscesses which had no relation to caseation. They were simply masses of leucocytes surrounded by granulation tissue. This granulation tissue, richly vascularized, had only a minor infiltration with epithelioid cells, and there were but a few small clumps of epithelioid cells in the neighborhood.

Rabbit R 613 showed the same extensive formation of specific tubercular tissue, widely distributed in the peritoneum, but especially massive in the mesentery and the omentum. Figs. 16 and 17 on Plate 5 show the reaction in the omentum. In Fig. 16 are three tubercle-like bodies with two large giant cells, probably of the rosette type, but possibly atypical, through the massing of the nuclei at one end. There is a dense capsule of lymphocytes. Fig. 17 shows tubercular granulation tissue, more dense in the lower part of the section. Along the upper border is a tiny, tubercle-like clump of epithelioid cells, and on the left border is a typical rosette giant cell. The tissues of Rabbit R 614 are represented in Fig. 18, which again shows small and larger collections of epithelioid cells with some diffuse infiltration of lymphocytes. In this animal the tissue was especially massive on the posterior body wall and around the reproductive organs. There were more epithelioid giant cells in this animal, but never as many as with the phosphatide.

It will be seen that all three of the rabbits receiving twelve doses of the fatty acid I, diluted with a bland oil, showed an extensive production of tubercular tissue, some of it as massive as with the phosphatide, but much more in the form of clumps of epithelioid cells, making circumscribed, tubercle-like formations, similar to those of the infection. There was only a moderate tendency toward epithelioid giant cells. There were some tubercular granulations, but a comparison of Plate 5 with the plates of tissues from the phosphatide will indicate that there was less variation in the type of specific reaction with this fatty acid than with the phosphatide, that is, the lesions were more uniform. There was, of course, a considerable increase in non-specific connective tissue, to make a framework for the tubercle-like structures, but this interstitial tissue lacked the extreme reaction found with the undiluted acid. In general, this fatty acid gave a reaction, when its irritative effects were minimized by dilution in mineral oil, which simulated the actual disease more closely than any other part of the lipoids; it was almost the same as the phosphatide from which it was derived, in epithelioid formation, but had more of the reaction in the form of discrete tubercles.

Effects of the Purified Wax and Its Derivatives

In Table I it will be seen that there were two groups of fractions which were soluble in chloroform and insoluble in acetone. Dr. Anderson has designated these substances "purified wax" and "soft wax," though he does not regard them as true waxes in the chemical sense (9).

The purified wax is a white, very light powder. From it Dr. Anderson obtained an unsaponifiable substance and a second liquid, saturated fatty acid. All three of these fractions had to be given in mineral oil. The purified wax could not be rubbed into a suspension in distilled water, as was done with the phosphatide; even less could the unsaponifiable substance, which floated as a dry, feathery substance on top of the water. Both of them were readily soluble in mineral oil. The purified wax and its fatty acid II gave reactions which we estimate as being positive for the specific formation of tubercular tissue; nevertheless, the reaction was smaller in amount than that of the phosphatide and fatty acid I. We estimate the amount of this reaction as ++ in comparison with the ++++ of the phosphatide. It was therefore necessary to make a careful comparison of the reaction of the purified wax and fatty acid II with that from the injection of the mineral oil alone. Beside this moderate reaction toward monocytes, epithelioid cells, and Langhans' giant cells (Plate 6, Figs. 20–23), the purified wax and fatty acid II gave a marked increase in general connective tissue, which is also found in tuberculosis. The unsaponifiable substance gave only an increase in general connective tissue cells.

The Purified Wax.—Five rabbits were tested with the purified wax intraperitoneally, as is shown in Table V.

Rabbit R 455 had one dose of 20 mg. of the purified wax, with 0.75 cc. of oleic acid as a diluting menstruum, and the tissues were studied 2 days later. The result was a marked irritation, and the tissues showed many abscesses. There was some phagocytic activity of the clasmatocytes and in the supravital studies some monocytes were seen, but they were few compared to the leucocytes. Oleic acid alone was found to be very irritating to the tissues and so was abandoned as a diluting menstruum.

Given in mineral oil, the material was much less irritating; in rabbits with few doses, R 550 with two, and R 551 with three, there were some neutrophilic leucocytes and some activity of clasmatocytes in destroying them, but there was a striking increase in monocytes, with a few epithelioid cells in R 551, and this strain of cells showed active division in sections. This tissue on the whole did not simulate that of tuberculosis, and yet the increase in monocytes and the occasional epithelioid cell would be described as the most striking reaction.

Two rabbits, R 937 and R 938, received twelve intraperitoneal doses of 16 mg. of the purified wax in 0.5 cc. of mineral oil. At autopsy, 24 hours after the last dose, a marked thickening of the omentum was found; the diaphragm, the capsules of liver and spleen, and the wall of the cecum were also involved. In Rabbit R

TABLE V

Protocols

Effects of Intraperitoneal Injections of the Purified Wax and Its Derivatives, the Unsaponifiable Substance and Fatty Acid II

No. of rabbit	Dose	Diluting menstruum	Results
		Purified wax	
R 455	1 of 20 mg.	0.75 cc. oleic acid	Marked irritation, peritonitis; many white nodules of pus. Clasmatocytes active in phagocytosis; many monocytes
R 550	2 of 16 mg.	0.5 cc. mineral oil	Omentum stimulated; some free neutrophilic leucocytes, many engulfed in clasmatocytes; many monocytes, scattered and in small clumps in active division. A few epithelioids
R 551	3 of 16 mg.		Omentum stimulated; more free leucocytes than in R 550. Clasmatocytes active. Many monocytes in division. Occasional epithelioid giant cell
R 937	12 of 16 mg.		No peritonitis; serosa smooth and glistening. Omentum, surface of diaphragm, and capsule of spleen massively thickened. Tissue edematous, red with white spots; looked like tuberculosis. Marked increase in general cellular connective tissue, with a few tubercle-like bodies and typical Langhans' giant cells. Some foreign body giant cells. A few plasma cells
R 938			No peritonitis; serosa smooth and glistening. Distribution of new tissue as in R 937. Omentum massive with general connective tissue. A few nodules of monocytes. Many cells difficult to classify because they have engulfed so much oil. Tissues look more like reaction to unsaponifiable substance
		The unsaponifiable substance	
R 548	2 of 16 mg.	0.5 cc. mineral oil	Serosa smooth and glistening. Omentum not thickened but showed both neutrophilic and eosinophilic leucocytes; some phagocytosis of them by clasmatocytes. A few monocytes
R 549	3 of 16 mg.		No peritonitis. Omentum normal, except that serosal cells were stimulated. Some milk spots increased in density. No leucocytes. Few monocytes

TABLE V—*Concluded*

No. of rabbit	Dose	Diluting menstruum	Results
		The unsaponifiable substance—*Concluded*	
R 945 R 946	12 of 16 mg.	0.5 cc. mineral oil	No peritonitis. Marked increase in connective tissue in omentum, capsule of liver and spleen, and over the diaphragm. Few free leucocytes in R 945; many clumps of lymphocytes; many monocytes; no epithelioid cells; moderate number of foreign body giant cells. Very few Langhans' giant cells. Few plasma cells
		Fatty acid II	
R 539	2 of 16 mg.	0.5 cc. mineral oil	Omentum showed accentuation of milk spots due to emigration of leucocytes and maturation of monocytes
R 540	3 of 16 mg.		Omentum: large dense milk spots due to emigration of leucocytes and to monocytes, epithelioid cells, and few epithelioid giant cells. Occasional foreign body giant cell. Marked dilatation of vessels. Sections show some clumps of monocytes
R 615	12 of 16 mg. every 24 hrs.		Parietal peritoneum, omentum, mesentery, and capsule of spleen and liver massively thickened. Omentum so cellular and dense, difficult to make films. Many clumps of monocytes with occasional epithelioid cell. Typical Langhans' giant cells
R 616	12 of 16 mg. every 48 hrs.		Omentum shows large milk spots in central area; rest massively thickened. Leucocytes and stimulated clasmatocytes. Clumps of cells were predominately epithelioid cells, rather than monocytes as in R 615

937, the tissue had, in the gross, the appearance of tuberculosis; it was swollen and edematous, was pink in color, and showed many white spots. In supravital preparations it appeared that there had been a marked new formation of undifferentiated connective tissue cells; these were of medium size, round, and showed less reaction to neutral red than do fibroblasts, clasmatocytes, or monocytes. Beside these undifferentiated cells, there had been a considerable development of epithelioid cells with the typical rosettes of fine bodies and many Langhans' giant cells, which predominated in number over the foreign body giant cells which were also present. There was an increase in young monocytes and the amount of the

specific reaction, that is, of epithelioid cells and epithelioid giant cells, was sufficient to place the purified wax in the group of the active lipoidal substances.

The tissues of Rabbit R 938 were likewise much thickened; supravitally the reaction was mixed and the specific reaction less in amount. Sections of the material showed a great increase in new indifferent connective tissue cells; these appeared as masses of nuclei without distinct cellular outlines. The masses of nuclei were taken to represent the clumps of undifferentiated cells in the fresh preparation, where the cellular outlines were sharp. Such masses of nuclei under low power suggest tubercles only vaguely and at higher powers not at all. In general the sections would not have been called characteristic of tuberculosis, because the epithelioid cells, both those that occurred alone and those in small clumps, were so scattered, and the indifferent connective tissue with a great increase in nuclei predominated to such an extent. Giant cells numbered from ten to twenty per low power field of the microscope. Some of them were typical of the rosette type with peripheral nuclei, but more of them looked like the non-rosette forms; most of them had taken in large or small droplets of the mineral oil which made them atypical. The epithelioid cells were in part typical, as was seen by the supravital technique, when their rosettes had the fine bodies, but many of them in sections appeared vacuolated, probably also from the presence of the mineral oil in medium sized droplets. There were many scattered monocytes. In places the small clumps of epithelioid cells were surrounded by lymphocytes; in others lymphocytes were lacking. On the diaphragm there were several quite definite tubercle-like structures. In general, though there was distinctly more of a reaction toward the formation of epithelioid cells and Langhans' giant cells than in the controls with mineral oil alone, this portion of the reaction was minimal as contrasted with the production of indifferent cells in the connective tissue itself. The sections from Rabbit R 938 were similar to those from R 937, except that they showed fewer epithelioid cells and giant cells.

The reaction to the purified wax was thus considered to be ++ in contrast to the ++++ of the phosphatide, as far as the production of epithelioid cells and Langhans' giant cells was concerned. The reverse would be the case if the amount of increase in general cellular connective tissue were appraised. The predominant reaction was of indifferent connective tissue cells, and there was in both rabbits, R 937 and R 938, a diffuse reaction of plasma cells in moderate numbers.

The Unsaponifiable Substance.—The unsaponifiable partition of the purified wax—the acid-fast portion of the lipoids—also showed the tendency to cause a marked increase in general connective tissue cells, without any of the stimulus toward the formation of tubercle-like masses of epithelioid cells.

The unsaponifiable substance was given to four rabbits (Table V). Rabbits R 548 and R 549, which received two and three doses of 16 mg. in 0.5 cc. mineral oil, showed only a slight reaction; in both the serosal cells appeared stimulated in the fresh preparations, that is, they showed an increase in small refractile bodies. There were free leucocytes in R 548, both neutrophilic and eosinophilic, and the clasmatocytes showed a slight increase in neutral red bodies; a few clasmatocytes contained leucocytes. Some of the milk spots were almost wholly of monocytes, and sections showed them in clumps, together with a few smaller, more basophilic mononuclear cells. There was an occasional mononuclear cell in division. This reaction was negative for tubercular tissue.

Two rabbits, R 945 and R 946, were then given twelve doses of 16 mg. of the unsaponifiable substance in 0.5 cc. mineral oil, and were studied 24 hours after the last dose. In both animals there had been a marked increase in new tissue, involving the peritoneum in the same areas as with the phosphatide, that is, the omentum, the capsule of liver and spleen, and the diaphragm. The new tissue equalled in amount that produced by the phosphatide; the omentum was just as massive. The cellular reaction, as seen supravitally and in sections was, however, quite different. There was a marked increase in new connective tissue cells. The milk spots were predominantly of medium sized cells, with a few scattered neutral red bodies. There were many monocytes. Sections showed an increase in general connective tissue cells, with replacement of the fat, and scattered everywhere among these less differentiated cells were diffuse masses of young, very basophilic, mononuclear cells, together with some monocytes and a few plasma cells. There was a moderate amount of cell division in the mononuclear forms. There were a few scattered giant cells, mainly of the foreign body type.

Thus the unsaponifiable material caused a marked increase in nonspecific connective tissue cells with some increase in monocytes, but did not produce the specific tubercular reaction of epithelioid cells and epithelioid giant cells.

Fatty Acid II from the Purified Wax.—The fatty acid II caused the production of tubercular tissue in about the same amount as the purified wax, indicating that the entire active principle of the purified wax was carried in this component. The reaction has therefore been designated ++.

The acute reaction was tested in two rabbits, R 539 with two doses of 16 mg. in 0.5 cc. mineral oil, and R 540 with three (Table V). The reaction was of leucocytes and monocytes. The peritoneal exudate reflected this; for example, in R 539, the percentage of leucocytes was 26, of monocytes 68, with the remaining 6 per cent of lymphocytes and serosal cells. The rabbit with three doses, R 540, had a few epithelioid cells among the monocytes.

Two rabbits, R 615 and R 616, were given twelve doses of 16 mg. of fatty acid II in 0.5 cc. mineral oil, the first every day and the second every other day. In both there was a marked increase in new tissue in omentum, mesentery, and under the parietal peritoneum. The omentum was fully as massive as with the phosphatide; there was almost no exudate. The tissue from these rabbits differed from that with the phosphatide and fatty acid I in that only a part of the new tissue was of monocytes and epithelioid cells, and there was a great reaction of non-specific connective tissue cells, undifferentiated in type, and fibroblasts. Thus, as can be seen on Plate 6, Fig. 20, the tissues do not suggest tuberculosis as conclusively as does the reaction to fatty acid I (Plate 5) or to the phosphatide (Plates 1–4). Fig. 20 was taken with a high dry lens at a magnification of 200 It can be seen that the clumps of monocytes and epithelioid cells are so tiny that they would not be conspicuous under a low power lens. The reaction is, however, of typical tubercular granulation tissue. There are scattered lymphocytes, but none in capsules around the tiny clumps of monocytes. The vessels at the right are crowded with lymphocytes. Scattered everywhere throughout this tissue were clumps of cells which on supravital examination were called monocytes, with an occasional epithelioid cell, and a few epithelioid giant cells. This is exactly what can be seen in Fig. 21. Except for the two larger cells, probably epithelioids, in the lower left border and a small Langhans giant cell in the center, this section shows a clump of monocytes; above this is a small epithelioid giant cell and below the clump an especially typical form. In Fig. 22 is another typical epithelioid giant cell, with a central area of necrosis, and the débris of a leucocyte; such an area of necrosis is frequent in the corresponding giant cells of the actual infection. The infiltration of these tissues with monocytes is shown in Figs. 22 and 23. In the latter, one of the cells, probably a monocyte, is in division. The amount of non-specific connective tissue is best seen in Fig. 20. The tissues of Rabbit R 616 were similar, except that there were fewer young monocytes and the tiny clumps of cells, microscopic tubercles, were epithelioid types; in this connection it will be noted that the reaction was of longer duration in this animal, since the injections were given every other day.

Thus, all of the three substances in this group, the purified wax and its derivatives, the unsaponifiable substance and the liquid fatty acid II, contain a stimulus for the production of relatively undifferentiated, connective tissue cells. This is the dominant reaction. Beside this increase in connective tissue cells, the purified wax and the fatty acid II produce some epithelioid cells, showing a rather marked tendency to cause a formation of tiny clumps or tubercles, which, however, are not surrounded by lymphocytes to any extent. Epithelioid giant cells are numerous and typical of those found in the actual infection. The relative amount of this specific tubercular reaction

from the purified wax and fatty acid II may be estimated as ++; and from the unsaponifiable substance as zero.

Effects of the Soft Wax and Its Derivative Fatty Acid III

The "soft wax," which is soluble in chloroform and insoluble in acetone, is a brown, wax-like substance, a soft solid at room temperature, miscible with mineral oil. It yielded on chemical analysis (Anderson, 9) a liquid, saturated fatty acid which has a deeper yellow color than fatty acid I.

The soft wax was given to four rabbits (Table VI). In the acute reaction (Rabbit R 546, two doses of 16 mg. in 0.5 cc. mineral oil, and R 547, three doses) the soft wax proved to be much more irritating than the purified wax. After two doses the serosal cells were stimulated, as shown by refractile bodies in them; the predominating reaction was of leucocytes with clasmatocytes engulfing them. After three doses the serosal cells were filled with refractile bodies and made so dense a membrane that it was hard to focus through them in fresh preparations. In both animals there was a marked dilatation of the blood vessels. After two injections the tissues were massive with leucocytes, but there were some monocytes; after three doses there were fewer leucocytes and more monocytes.

Two rabbits, R 947 and R 948, were given repeated injections. Rabbit R 947 was found dead of pneumonia after the tenth dose; the other one was given twelve doses of 16 mg. each in 0.5 cc. mineral oil. In Rabbit R 947 there was a fresh peritonitis, especially evident over the liver and spleen. Rabbit R 948 showed no infection. In both animals there was a great dilatation of the blood vessels in the new tissue in the omentum and over the abdominal organs. Fig. 24 on Plate 7 is taken from extensive lesions on the abdominal surface of the diaphragm. The lesion measured 2 mm. in thickness in the sections which had undergone the shrinkage of the dehydration. The dilatation and congestion of the veins are a striking factor, as is shown in the photograph. There are two small clumps of epithelioid cells; the larger is at the upper left corner and is infiltrated with lymphocytes, and the smaller is at the lower left corner. The section also showed scattered epithelioid cells. Giant cells were present in the tissues in moderate numbers, some of them typical rosette types; others of the non-rosette type with large vacuoles.

There was a considerable new growth of connective tissue, not as marked, however, as with the purified wax and its derivatives. For example, the fat of the omentum was only partially replaced by connective tissue cells. There was some typical granulation tissue, with many fibroblasts. There were many leucocytes and many of them

TABLE VI

Protocols

Effects of Intraperitoneal Injections of the Soft Wax and Its Derivative, Fatty Acid III

No. of rabbit	Dose	Diluting menstruum	Results
			Soft wax
R 546	2 of 16 mg.	0.5 cc. mineral oil	Milk spots of omentum markedly stimulated. Predominating reaction of leucocytes scattered and in small foci. Many clasmatocytes full of yellow débris. Serosal cells full of refractile bodies. Some monocytes. Vessels dilated
R 547	3 of 16 mg.		Some seropurulent fluid. Omentum: extensive stimulation of milk spots and fat replaced; leucocytes; serosal cells markedly irritated. Some monocytes and epithelioid cells in small clumps. Marked dilatation of vessels
R 947	10 of 16 mg.		Died of pneumonia night after 10th dose. Body wall had hemorrhage; no puncture of intestine; marked peritonitis with general fibrinous exudate. Omentum moderately thickened, leucocytes and small tubercle-like clumps. Marked dilatation of vessels
R 948	12 of 16 mg.		Omentum much thickened; diaphragm, liver, and spleen covered with new tissue; extreme dilatation and congestion of vessels (Fig. 24). Many scattered epithelioid cells and tubercle-like structures. Lymphocytes
			Fatty acid III
R 542	2 of 16 mg.	0.5 cc. mineral oil	Omentum: slight increase in milk spots but no thickening. No peritoneal irritation. Few leucocytes, neutrophilic and eosinophilic; predominating reaction clasmatocytes engulfing leucocytes. Few monocytes. No epithelioid cells seen
R 543	3 of 16 mg.		Omentum delicate; no marked thickening; very few leucocytes free and only an occasional one in clasmatocytes. Marked monocytic reaction, many in division. Few epithelioids

TABLE VI—*Concluded*

No. of rabbit	Dose	Diluting menstruum	Results
		Fatty acid III—*Concluded*	
R 617	12 of 16 mg.	0.5 cc. mineral oil	Adhesions between cecum and body wall. Some thick, ropy, peritoneal exudate. Omentum massively thickened. Leucocytes not marked; few in clasmatocytes. Predominating reaction of monocytes, epithelioid cells, and Langhans' giant cells. Few plasma cells
R 618			Hemorrhage into body wall. Cecum adherent to body wall. Omentum extremely thickened; marked dilatation of vessels. Mesentery also involved. Clasmatocytes active but predominating reaction of clumps of monocytes and tubercle-like masses of epithelioid cells. Few giant cells. Lymphocytes. Many foci of basophilic mononuclear cells. Few plasma cells

were in clasmatocytes. In Rabbit R 947 there was a focus of myelocytes in the omentum. Scattered through this tissue were considerable numbers of epithelioid cells; especially over the intestine and the diaphragm there were many more epithelioid cells than in the reaction to the purified wax. Giant cells were present in small numbers, some epithelioid in type, more of the large, multinuclear foreign body types. The reaction includes positive specific tissue which is intermediate in amount between that found following the purified wax and the phosphatide.

Fatty Acid III from the Soft Wax.—Four rabbits were given the fatty acid III, two for the acute reaction (R 542 and R 543) and two (R 617 and R 618) for the effect of twelve repeated doses (Table VI).

After two doses (R 542) the milk spots of the omentum were increased in number but were not much thickened. The tissues showed neutrophilic and eosinophilic leucocytes and some phagocytosis of these by clasmatocytes, and there was an increase in monocytes. Sections showed a marked activity of the clasmatocytes in destroying leucocytes, and there was much cell division both of the serosal cells and of free mononuclear cells, the size of monocytes. The monocytes and phagocytic clasmatocytes were plain both in the folds of the omentum and in the exudate caught between these folds. In Rabbit R 543 the omentum was more thickened;

clasmatocytes were still present, containing leucocytes, but the monocytic reaction had become predominant. The supravital reaction revealed as pure a monocytic response as with any of the lipoid fractions; this reaction was clear in the sections, making excellent tissue for the study of this type of mononuclear cell.

Rabbit R 617 received twelve intraperitoneal doses. There was a marked development of new connective tissue; the non-specific connective tissue was less cellular than that produced by the purified wax and its derivatives, but scattered everywhere in it were tubercle-like structures, similar to those from fatty acid II, but more in number and somewhat larger. Some of these clumps of epithelioid cells were densely surrounded by lymphocytes. There were few giant cells, small rosette forms and large foreign body types.

Rabbit R 618 showed the same reaction, but the clumps of epithelioid cells were still larger and more numerous. These lesions are shown in two photographs (Plate 7, Figs. 25 and 26). Multiple tubercle-like structures stained rather faintly are shown in Fig. 25, with a mass of lymphocytes at the upper right border. Fig. 26 is from the mesentery near the mesenteric lymph node; it shows a clump of epithelioid cells and one large giant cell, with a few epithelioid cells near it. The giant cell is complex. It has a peripheral row of nuclei and at one end a clump of nuclei; it had phagocytized a leucocyte and in one zone the cytoplasm is vacuolated. It is probably an atypical epithelioid or Langhans type.

In general the specific reaction caused by fatty acid III was overwhelmingly toward epithelioid cells as compared with giant cells. Only a few giant cells were found; in some sections none. The specific activity of the soft wax and of the fatty acid III derived from it was practically equal in amount, and can be considered as +++ in comparison with that of the phosphatide (++++) and fatty acid II (++).

Glycerides

The acetone-soluble glycerides (Table I) also soluble in alcohol-ether, have not been tested biologically, but we have studied the effects of the saturated fatty acids derived from the glycerides. The mother substance was a brown solid of wax-like consistency. It yielded on analysis two saturated fatty acids, designated IV (Anderson's No. 1) and V (Anderson's No. 2), in Table I. The proportion of fatty acids in the original glyceride, namely, about 40 per cent, was much higher than that of any of the other three primary lipoids. Because of the larger quantities available, Dr. Anderson has been able to separate these two fatty acids by fractional distillation, and has found that each of them is composed of the same two simpler fatty acids, one optically

active and the other optically inactive, only combined in different proportions. He considers that the fatty acids previously described, I, II, and III, are also mixtures of fatty acids which may be separated by fractional distillation when sufficient quantities are available. Fatty acid IV contained more of the optically inactive form, which, on analysis, proved to have the formula $C_{18}H_{36}O_2$. This isomer of stearic acid, Dr. Anderson has named tuberculo-stearic acid. It is an oil at room temperature.

Fatty acid V contained a greater proportion of the optically active acid of the formula $C_{26}H_{52}O_2$. Dr. Anderson has named this fraction phthioic acid. It has a low melting point, 28°C., and is optically active; $[\alpha]^{20} = 7.98°$. The tuberculo-stearic and phthioic acids represent the highest chemical purity of any of the fractions thus far tested.

Fatty acids IV and V from the glyceride, and the phthioic acid all produced epithelioid cells and epithelioid giant cells in moderate numbers, while the tuberculo-stearic acid was negative in this reaction, indicating that the active element in all three positive substances is the optically active phthioic acid. With these fatty acids, the epithelioid cells were scattered, as in Fig. 27, with no tendency whatever toward the formation of tubercle-like bodies. In this regard these acids have differed from the other liquid saturated fatty acids, from phosphatide, purified wax, and soft wax, with all of which tubercle-like bodies were formed. The epithelioid cells produced were, however, entirely typical if relatively few in number, as indicated by the symbol + in Table I.

The experiments with these two acids before and after the final purification are summarized in Table VII.

Two rabbits were given twelve intraperitoneal doses of each original mixture in 0.5 cc. mineral oil, but the dosage of the purified forms was in each case only 5 mg. in contrast to 20 mg. of the corresponding fatty acids before purification.

Rabbits R 918 and R 919 received twelve doses of 20 mg. of fatty acid IV. Rabbit R 918 had a great excess of adipose tissue in omentum and mesentery, and the omentum was only moderately thickened with new tissue. On the other hand, the omentum of Rabbit R 919 was markedly thickened. In both animals the most striking reaction was of giant cells, both types being present, but with more of the large, non-rosette types. Both showed some scattered epithelioid cells, none in the form of tubercles, not even in small clumps like those from the purified wax.

TABLE VII

Protocols

Fatty Acids from the Glycerides

No. of rabbit	Substance tested	Dose	Diluting menstruum	Results
Fatty acid IV and its major fraction, tuberculo-stearic acid				
R 918	Fatty acid IV	12 of 20 mg.	0.5 cc. mineral oil	Great excess of adipose tissue in peritoneal cavity; omentum only moderately involved with new tissue. Few scattered epithelioid cells showing division; many giant cells, both types, predominantly foreign body; some free leucocytes; plasma cells. Marked dilatation of vessels
R 919				Omentum markedly thickened; few scattered epithelioid cells; many giant cells, predominantly foreign body types; some free leucocytes; plasma cells; marked dilatation of vessels; clumps of myelocytes in omentum
R 980	Tuberculo-stearic acid	12 of 5 mg.		Omentum moderately thickened; milk spots of undifferentiated cells showing less reaction to neutral red than monocytes; occasional epithelioid cells; many foreign body giant cells; some free leucocytes; plasma cells
R 981				Omentum moderately thickened, weight 7 gm.; milk spots showed monocytes and occasional epithelioid cells; many foreign body giant cells; few free leucocytes; plasma cells
Fatty acid V				
R 920	Fatty acid V	9 of 20 mg.	0.5 cc. mineral oil	Died; immediate autopsy. Cause of death not determined. Cirrhosis of liver, large spleen (3 gm.); great excess of adipose tissue in peritoneal cavity; omentum showed some stimulated areas filled with cells, nature of which not determined, size of epithelioids but loaded with refractile bodies

TABLE VII—*Concluded*

No. of rabbit	Substance tested	Dose	Diluting menstruum	Results
Fatty acid V and its major fraction, phthioic acid—*Concluded*				
R 921	Fatty acid V	12 of 20 mg.		Omentum markedly thickened; serosal cells filled with refractile bodies; scattered epithelioid cells, in division, striking reaction; some epithelioid giant cells; few free leucocytes; plasma cells
R 982	Phthioic acid	12 of 5 mg.	0.5 cc. mineral oil	Omentum markedly thickened; clasmatocytes containing leucocytes or yellow débris; no peritonitis; monocytes predominating reaction with a few epithelioid cells; great increase in mononuclear free cells; many plasma cells
R 983				Massive thickening of omentum, weight 18.5+ gm.; many scattered epithelioid cells in active division; epithelioid giant cells; few leucocytes; plasma cells

With such a diffuse and slight reaction it is difficult to judge whether the increase was more than occurs with the mineral oil alone, but our estimate is that there was a slight specific reaction beyond that of the mineral oil. Both omenta showed some scattered leucocytes, some plasma cells, a marked formation of new blood vessels, and in the case of Rabbit R 919 much increase in non-specific connective tissue.

Rabbits R 980 and R 981 which received twelve doses of 5 mg. of the optically inactive tuberculo-stearic acid, showed a less marked reaction than the two rabbits just described, and markedly less than the two animals with corresponding doses of the optically active substance (R 982 and R 983). Rabbits R 980 and R 981 had only a moderately thickened omentum. Again the giant cells, which were largely of the non-rosette type, predominated over epithelioid cells. Epithelioid cells were in minimal numbers, estimated as not more than in the mineral oil controls. In Rabbit R 980 there was one structure which under low power looked like a tubercle, but under the oil immersion lens there were only a few typical epithelioid cells in it; the majority of the cells were highly vacuolated forms, with practically no dense cytoplasm. This structure was partly surrounded by lymphocytes. Thus we estimate that tuberculo-stearic acid has almost no specific

power in the production of epithelioid cells and only a little tendency toward the production of non-specific connective tissue, a finding in conformity with the reaction to commercial stearic acid itself. All four animals in this group showed plasma cells in considerable numbers. The fact that the dosage of the purified form was only one-fourth that of the fatty acid IV makes these conclusions subject to revision, if dosage subsequently proves to be a major factor.

Four rabbits (Table VII) received the optically active forms of the fatty acid from the glycerides. Rabbit R 920 (fatty acid V) proved to be unsatisfactory for the experiment on account of the great excess of adipose tissue in the peritoneal cavity. The animal died 2 days after the ninth injection, and the cause of death could not be determined at autopsy, which was made immediately after death. The omentum showed some stimulated areas in which the cells, as seen supravitally, were of the size of epithelioid cells, but were wholly filled with refractile bodies which in sections appeared as vacuoles. Their nature was not determined. The other three rabbits, R 921, R 982, and R 983, all showed a massively thickened omentum. The first one, R 921, received the fatty acid V (20 mg.) before the purification and the other two the smaller doses (5 mg.) of the phthioic acid. In Rabbit R 921 scattered epithelioid cells were a striking reaction with marked cell division, as is shown in Fig. 27. It will be noted in this section that there is practically no tendency toward the formation of tubercle-like clumps. Supravitally the milk spots of the omentum were seen to be filled with epithelioid cells. There were giant cells, many of them of the rosette type. There was also much non-specific connective tissue, with leucocytes, lymphocytes, and plasma cells. With the purified phthioic acid, the two animals differed in the specific response. In Rabbit R 982 the specific reaction was predominantly of monocytes, as seen in the fresh tissue, with only occasional epithelioid cells; in Rabbit R 983 there were more epithelioid cells, nearly as many as in Rabbit R 921. These cells were also in active division, so that Fig. 27 is a fair representation of them also. The comparison of the tissues of R 921 and R 983 is difficult because there was so much greater increase in the non-specific connective tissue in Rabbit R 983 than in R 921, which makes the epithelioid cells in R 983 appear more diffuse. If they are estimated as approximately the same in amount, the purified form of the fatty acid would be considered the more potent, since the dose was one-fourth as large.

Both Rabbits R 982 and R 983 had a marked increase in free, round mononuclear cells, as distinguished from the fixed connective tissue cells. In this regard the tissue is to be contrasted with that from the reaction to the purified wax and its unsaponifiable derivative, with which the non-specific connective tissue was composed more of fixed than of wandering cells. The free mononuclear cells in R 982 and R 983 were of varied types, lymphocytes, monocytes, and plasma cells. Leucocytes in considerable numbers were diffusely scattered in both omenta.

In Rabbit R 983 there was a very great increase in non-specific connective tissue; after the supravital preparations had been made, the omentum weighed 18.5 gm., as contrasted with the weight of the omentum in Rabbit R 981, namely

7 gm. We had not weighed the omenta from the rabbits in our series previously and the varying amount of adipose tissue would make the weights in normal controls subject to a wide variation. But in a few controls which had been given relatively inert substances and had relatively little excess fat, the weights have varied between 1 and 4 gm. In Rabbit R 983 the adipose tissue had been markedly replaced by new connective tissue, so that the former was not a disturbing factor in the weight of 18.5 gm. which may thus be considered as indicating a marked increase in new tissue. In this new tissue the specific reaction is minimal as compared with the non-specific.

In all the animals receiving the derivatives of the glycerides, the diffuse character of the reaction of epithelioid cells stands in contrast to the clumping of epithelioid cells, already described in the case of the phosphatide, and of the waxes and the fatty acids derived from them. Plasma cells have been a constant reaction to all the preparations of fatty acids from the glycerides and have thus been a more consistent response in this group than in any of the other fractions tested. In general, we have not found plasma cells in the acute reactions, those after one, two, and three injections, but they have been present occasionally after the twelve injections.

SUMMARY

The fatty acid I from the phosphatide proved to be too irritating for administration undiluted, but dissolved in a bland oil it produced tubercular tissue in approximately the same amount as the phosphatide and in tubercle-like masses that closely resembled the reaction of the actual infection. Its amount is indicated as ++++. The chloroform-soluble lipoids insoluble in acetone, which were in the form of wax-like substances, and the liquid saturated fatty acids derived from them caused the production of abundant epithelioid cells and epithelioid giant cells, in the form of tubercle-like bodies. The specific activity of the soft wax and fatty acid III might be estimated as +++; of the purified wax and fatty acid II as ++. The soft wax was more irritating than the purified wax and caused much more congestion; the purified wax and the unsaponifiable partition from it gave a marked increase in general connective tissue cells.

The original glyceride fraction has not been tested; it contained two fatty acids (IV and V), in much higher proportion (40 per cent) than the phosphatide A-3 and the waxes. Each of these fatty acids was

found to be a mixture of two saturated fatty acids; one, optically inactive, an isomer of stearic acid, and therefore called tuberculo-stearic acid, was relatively inert in the tissues; the other, optically active, and named phthioic acid, caused a production of scattered epithelioid cells but not in aggregates like tubercles. It is probable that the saturated fatty acids from the phosphatide A-3 and from the waxes also contain tuberculo-stearic and phthioic acids in varying proportions.

NON-SPECIFIC REACTIONS TO LIPOIDS, AND CONTROLS

The partitions of the lipoid which have proved inert as regards specific ability to produce epithelioid cells are considered in this section, as are also certain other substances used either as diluting menstrua for the fatty acids, or as general controls.

For the study of the direct effect of any substance upon the connective tissues, the intraperitoneal route has certain advantages owing to the nature of the omentum.

This broad, thin membrane affords a most favorable place for the study of the cells comprising the diffuse connective tissues, inasmuch as any substance injected into the peritoneal cavity passes directly to it and affects its elements. From the experimental standpoint, the omentum may be thought of as offering an opportunity to study tissue reactions occurring *in vivo* in a manner somewhat analogous to the method of *in vitro* tissue culture. It does not offer, of course, the opportunity to observe cells under conditions of strict isolation (tissue culture) over a period of days, nor that of experimenting on such strains *in vitro*. On the other hand, it does give opportunity to analyze reactions in a relatively simple background, while the cells are at the same time subject to the factors of general metabolism and physiological environment of the organism as a whole. The analogy to tissue culture rests, of course, on the nature of the milk spots, which are small clumps of young cells that, in the early response to certain stimuli, grow as an almost single strain of cells and radiate out from the edges of the central mass into the membrane so as to simulate the appearances of a culture. Beside this striking reaction within the milk spots, the more general response of the omentum involves an outpouring of cells from blood to tissues, changes in the blood vessels themselves, and changes in the general connective tissue cells of the organ. These cellular activities may be in the nature of increased division, or increased phagocytic action of mature cells, or of maturation of new forms from primitive types, or the formation of cell types which may be considered as pathological. Any specific reaction must be analyzed in terms of all these factors.

Normal Omentum and Peritoneal Fluid.—A study of the normal omentum and peritoneal fluid was a necessary background for our experimental work. There have been three recent surveys of the omentum and its cells (von Möllendorff, 20, Cunningham, 21, and Maximow, 22). For the purposes of the studies here reported, it has been necessary to establish the normal under the same conditions and with the same technique used in testing the fractions from the tubercle bacillus. This has consisted in a supravital study of the living cells of the omentum and peritoneal fluid stained in neutral red and Janus green, the same technique used by Cunningham, correlated with a study of fixed sections and fixed films of the cells.

As is well known, the normal omentum is a thin, double layer of delicate connective tissue in which may be seen a few sparsely scattered, small, opaque areas, the milk spots. When the two layers are carefully separated and one thickness is spread on a slide, it appears to be an intact membrane with complete covering of serosal cells. With the slightest irritation the serosal cells develop refractile bodies and tend to drop off, leaving denuded areas. Mounted on a film of neutral red, the serosal cells show no reaction to the dye and the fibroblasts but little reaction; in each field there are a few scattered, elongated clasmatocytes or macrophages which contain scattered, small masses of débris staining a deep red in the dye. These cells comprise the principal stained elements of the interspaces.

The milk spots are small clumps of cells, sometimes not more than 20 in number. In eleven animals in which the omentum was, as far as could be determined, entirely normal, two types of cells predominated in the milk spots. One of these was the cell we have called the monocyte with typical rosette of neutral red bodies, and the other a cell of like size with neutral red bodies like those of the rosette of the monocyte, but so scattered in the cytoplasm that they do not accentuate the area of the centrosphere. A given milk spot may be entirely of rosette cells, or of cells with scattered neutral red bodies, or mixed. In one rabbit (R 651), the milk spots were densely cellular and made up of round types the size of monocytes or larger, with a dense, uniform cytoplasm that showed scarcely any reaction to neutral red. The nuclei of these cells were large, round, centrally placed, and had conspicuous nucleoli. These cells we consider as more primitive than the rosette cell or the type with scattered neutral red bodies, and not to be identified as monocyte, clasmatocyte, or lymphocyte. It is clear that the study of living connective tissue has for its object the detection of signs of immaturity and differences in function or in functional states within the group of connective tissue cells. Nagel (23) has recently found that the fibroblasts in tissue culture show but little reaction to neutral red in young (24 hour) cultures, while the cells of the more mature cultures stain well.

TABLE VIII

Differential Counts of Cells in Peritoneal Fluids

Substance injected	None		Distilled H_2O	Normal saline	Mineral oil	Palmitic acid in mineral oil	Stearic acid in mineral oil	Sodium glycero-phosphate in H_2O	Glycerol in mineral oil		Red phosphorus in saline	Polysaccharide	Protein 304-F
Number of rabbits used	11		1	1	4	2	3	2	2	1	1	2	1
Number of injections	None		12	12	12	12	12	7 and 12	2	12	1	12	12
	Average	Range											
Total cells per c.mm	2,285	175 to 8,000	12,700	25,200				Few					
Neutrophilic leucocytes	1.5%	0–3%	2%	3%	25%	7%	13%	13%	32%	3%	44%	51%	70.5%
Lymphocytes	18	2–56	26	16	22	3	10	7	4	1	0	7.5	3
Monocytes	60	10–87.5	63	73	46	54	72	65	60	66	39	33	5.5
Clasmatocytes	9.5	5–36	9	6	3	5	3	9	1	1	17	3.5	20.5
Serosal cells	6	0–31.5	0	1	1	20	2	1	1	1	0	5	0.5
Unclassified	5	0–26*	0	0	2	0	0	5	1	28†	0	0	0

* Range 0 to 7 except for Rabbit R 651 with 26 per cent primitive cells.

† These cells (R 640) were of medium size and filled with refractile bodies.

Three of the eleven animals showed small foci of lymphocytes in the milk spots. Beside these strains of cells, an occasional milk spot contains a few long, branched clasmatocytes like those of the interspaces or a round phagocytic cell with the varied vacuoles characteristic of the active clasmatocyte; but in general the milk spots are made of cells that we consider as relatively young, for they are the first to respond with active cell division under experimental stimuli.

The cells of the peritoneal fluid were studied in the same eleven animals with total counts and a differential count made of the living cells. The results are averaged in the second and third columns of Table VIII. These figures are to be compared with the records of all the animals in this series in which counts of the cells of the peritoneal exudate were made.

In these counts the total free cells were below 2,970 per c.mm. in all but one rabbit, the range being from 175 to 8,000. It will be noted that the predominating form was the cell with the rosette, the so-called monocyte, while the more actively phagocytic forms containing irregularly staining débris which were recorded as clasmatocytes averaged 9 per cent. Only one basophil, no eosinophils, and only one monocyte with accentuated rosette were observed. Very few red blood cells were found—0 to 20 while counting 200 white cells.

In one rabbit (R 651) 26 per cent of undifferentiated cells were encountered, which corresponded with a predominance of the same type of cell in the milk spots of the omentum just described. Three of the animals showed definite foci of lymphocytes in the omentum, and in the respective peritoneal fluids these cells made up 34 (R 578), 48 (R 650), and 56 (R 879) per cent of the free cells found.

From these studies it may be accepted that there is so close a correlation between the omentum and the cells free in the peritoneal cavity as to suggest the omentum as the source of the free cells; that the milk spots are collections of young cells, more or less differentiated; that the phagocytic cell of the interspace is the clasmatocyte (macrophage or histiocyte); that the serosal cells and fibroblasts are inconspicuous in supravital films; and that the total number of free cells in the peritoneum is small. Gardner (24) has recently published figures on the normal differential count of the cells of the peritoneal fluid in guinea pigs, and his average and limits of variation for the different types closely parallel those here cited for the rabbit.

Effects of the Vehicles Used in Administering the Tuberculo-Lipoid

Water and Normal Saline.—In general two forms of diluting menstrua were obligatory in the fraction-testing experiments, aqueous for the phosphatide and a bland oil for the fatty acids and waxes. It

was obviously necessary to determine the effects of the menstrua as such.

There is an outpouring of neutrophilic leucocytes within 5 minutes after the injection of any fluid into the peritoneal cavity, a fact that has recently been emphasized again by Gardner. It has been necessary, therefore, to determine the state of the omentum after a series of injections of distilled water or of physiological saline, the diluents of choice for the introduction of any substance into the peritoneal cavity. In every instance the water employed in these experiments was freshly distilled from glass and sterilized immediately before using. From Table VIII it will be seen that the differential count of the free cells after injections of distilled water remained practically unchanged. Though the total cells per c.mm. showed a decided increase over the normal there was no percentage increase in neutrophilic leucocytes. In the animal (R 709) receiving the distilled water, the parietal and visceral peritoneum was entirely normal in appearance, except for a slight increase in vascular dilatation in the omentum. Microscopically there was evidence of a slight reaction, some of the serosal cells being rounded up with a few refractile droplets and the clasmatocytes with stained débris being slightly increased in number in the interspaces. The milk spots consisted, as normally, of monocytes and clasmatocytes, with an occasional monocyte having an increased rosette; no giant cells were seen. There were foci of both primitive cells and lymphocytes. No abnormal findings were recorded in any of the organs except in the bone marrow which showed supravitally a decrease in the usual fat content and on section revealed shrunken fat cells in a granular matrix. The myeloid and erythroid cells were entirely normal in their relationships. No anemia and no leucocytosis in the peripheral blood developed during the period of the injections.

In Rabbit R 730 the twelve daily injections of freshly prepared normal saline again increased the total number of free cells found in the peritoneal fluid without altering appreciably their normal percentages (Table VIII); the omentum showed no changes in the gross, and, supravitally, showed essentially the features mentioned as present after distilled water. In this animal, however, there was a sudden drop of 1,500,000 in the number of red blood cells per c.mm. and of 15 per cent in the hemoglobin (Newcomer) in the last 3 days of the injection period. At autopsy a local peritonitis at the site of injection over the wall of the cecum was encountered; this may have been responsible for the anemia. At no time was there found any increase of total white blood cells. The bone marrow was gelatinous and there was decreased fat content with a shift to earlier developmental forms in both red and white cell series.

It seems evident, then, that either the immediate increase of leucocytes in the peritoneal cavity initiated by the injection of salt solution or distilled water into the peritoneal cavity is transitory or, with repeated injections the response becomes minimal, because 48 hours

after the twelfth injection, neither peritoneal fluid nor omentum showed any increase over the normal in number of neutrophilic leucocytes. The fixed sections showed only scattered plasma cells and a few clumps of monocytes with an occasional epithelioid cell and clasmatocyte.

As has been explained in a preceding section (page 28), the specific, liquid, saturated fatty acids could not be given in water, and when given undiluted were so corrosive that any specific reaction was largely masked by an extreme irritation. Moreover, the waxes and their derivatives were not miscible with water, so an effort was made to find a bland oil as a menstruum. Olive oil, oleic acid, and two purified mineral oils were successively tested for their effect on the peritoneum in the hope that a relatively inert diluent might be found.

Olive Oil.—Five rabbits (R 338, R 341, R 665, R 800, and R 829) received olive oil intraperitoneally in daily doses of 5 cc. each. The severity and extent of the reaction varied somewhat in the several animals but even from the least reactive it was apparent that the irritative effect of olive oil was so great that it would mask effectively any specific reaction that might be expected from a substance given in combination with it. The effects observed ranged from a moderate omental reaction, consisting chiefly of many foreign body giant cells scattered singly and with little diffuse cellular reaction otherwise (R 341), through massive thickening with foreign body giant cells, clasmatocytes, and fibroblasts predominating (R 338), to a massive reaction involving monocytes, epithelioids, and epithelioid giant cells and foreign body giant cells in great abundance (R 829). In the last instance, in which trypan blue was given intravenously together with olive oil intraperitoneally, while there were areas suggestive of tubercular granulation tissue, the tissue in general did not show this reaction to the degree characteristic of the phosphatide A-3 and of the tuberculo-fatty acids. See protocols, page 57.

Oleic Acid.—Oleic acid was identified as one of the constituent fractions composing the original phosphatide A-3 of the tubercle bacillus and was considered as a possible menstruum for the introduction of other fractions. Six rabbits were given various dosages of the oleic acid. In all these there was a marked corrosive action on the peritoneal tissues. The smallest amount given—0.1 cc. in a single injection—was followed by a leucocyte-clasmatocyte reaction with abscess formation within 24 hours. Larger amounts over longer periods gave an accentuation of this reaction with foreign body giant cells and a marked non-specific irritative response.

It is thus clear that oleic acid plays only a non-specific rôle in tuberculosis if it is liberated in the tissues in the process of bacillary dis-

integration, but that inherent irritative properties eliminate it as a possible menstruum for other substances.

Mineral Oil.—A relatively pure mineral oil (Nujol) was next injected in a series of ten rabbits at different times and in different dosages, and, while again the reaction varied in different animals, it was found in general to be the least irritative of any of the solvents for lipoids tested. After from one to three injections of 0.5 cc. each the reaction was negligible. Three only of the eight rabbits receiving from ten to twelve injections of the mineral oil showed any considerable evidence of reaction at autopsy. All had appreciable quantities of the oil still free in the peritoneal cavity but in only one animal (R 663) was there any evidence of irritation other than in the omentum. In general the more striking effect was a non-specific stimulation of connective tissue involving clasmatocytes and fibroblasts. The nature of this reaction is shown in Fig. 19, Plate 6, which is a spread of the omentum from Rabbit R 661, stained with neutral red and photographed while the cells were still living. It shows a general increase in fibroblasts and in number and activity of elongated clasmatocytes of rather uniform type. This area is representative of the thinner portions of the omentum from this rabbit. In the thicker parts of the tissue the reaction was less uniform and showed granulocytes scattered diffusely, and a considerable number of foreign body giant cells. There was some monocytic response and a minimal reaction of epithelioid cells.

The minimal reaction observed after the use of this mineral oil alone established its value as a menstruum for introducing substances insoluble in water.

A still further purified, less viscous mineral oil (No. 4 Socony white oil, Standard Oil Company of New York), when given in one animal in the same repeated dosage as the Nujol, showed a reaction the equivalent of the least irritative response found after the latter, and can also be used as a diluent.

Special Controls for the Phosphatide Reactions

Killed Tubercle Bacilli.—The first reaction analyzed as an essential control was, of course, that elicited by the original killed tubercle bacilli themselves.

That dead tubercle bacilli produce tubercular tissue has long been known; the historical significance of this reaction has been traced in a previous report (3). The matter was conclusively settled in 1891 by Prudden and Hodenpyl (18, 19), but in their experiments injections of "2 to 3 cc. of a milky suspension" were used, from which the dose cannot be estimated, the experiments having been done before methods of weighing and counting of bacilli in suspensions had been em-

ployed. In the original phosphatide A-3, there was a small amount of material which was acid-fast and our first control was to inject a small dose of killed bacilli of the strain under investigation, H-37, in quantities (a total of from 3 to 7 mg. in fourteen doses) estimated to approximate the amount of acid-fast débris in a standard dosage of the phosphatide. The results of our experiments were reported in detail in 1927 (3). Small and infrequent tubercles were found proportional to the small dose of organisms but in no instance was there the quantitative reaction observed after the administration of phosphatide. As a further control, it was thought of interest to determine if possible the effect of heat-killed bacilli in dosage (1.6 gm.) the equivalent of the 80 mg. of the phosphatide A-3. In this dosage the phosphatide has been given in 10 cc. distilled water in twelve daily injections without any symptoms whatever. A single corresponding dose of 1.6 gm. of dead tubercle bacilli, H-37, killed each of two rabbits (R 1084 and R 1085) in less than 24 hours. The bacilli were found massed in the peritoneal cavity with relatively little absorption, the fatalities being due evidently to some soluble toxic products accompanying this large dosage.

Lecithin.—When it was found that the phosphatide A-3 gave such an extensive formation of epithelioid cells, another phospholipin, lecithin, prepared from brain tissue by Dr. P. A. Levene, to whom we are indebted for the material, was tested for comparison.

Two rabbits, R 243 and R 244, each received to begin with fourteen intraperitoneal injections of 80 mg. and then one of 122 mg. of lecithin each in 10 cc. distilled water. Though the amount was thus greater than that of the phosphatide A-3 from the tubercle bacillus, the cellular reaction was much less. In R 243 the omentum was almost normal in thickness in the gross, except that at one end there was a pink nodule which looked, with the unaided eye, like the reaction to phosphatide A-3. There were also one or two nodules in the mesentery. In R 244 much of the omentum was thin, but there were some pink bands too thick to study supravitally. The reaction was complex; there were many of the large, vacuolated cells, such as we have found after one, two, and three injections of the phosphatide A-3 (Figs. 1–3); but when these cells, which had stained brilliantly in neutral red, were faded in formalin and then restained with Sudan III, they gave the typical reaction of fat. In sections the reaction of the less dense areas might be described as a polymorphonuclear and round cell infiltration; there were clasmatocytes loaded with débris and with leucocytes, many free leucocytes, lymphocytes, and plasma cells, together with large, round, highly vacuolated cells, and a few scattered epithelioid cells. The nodules, on the other hand, which were seen as pink tissue in the fresh, proved to be typical, tubercle-like clumps of epithelioid cells with a few Langhans' giant cells; some of these foci were surrounded with lymphocytes and a few had been invaded with leucocytes.

The lecithin gave a mixed reaction with some production of tubercular tissue, typical in appearance, but minimal in amount, as compared with the reaction to the phosphatide A-3.

As a test of the biological reaction to *phosphorus*, one animal was given red phosphorus in normal salt solution with a marked granulocytic response, and some apparent formation of new young monocytes. Lawrence and Huffman (25) have observed an increase in the number of monocytes in the blood following six to seven subcutaneous injections of yellow phosphorus in oil.

Non-Specific Lipoid Fractions

The phosphatide A-3, Table I, yielded, upon analysis, four subfractions which did not produce epithelioid cells, and a fifth, a sugar acid which has not been tested. The non-specific irritant action of oleic acid has been described in a preceding section of this chapter, which dealt with the search for a bland menstruum in which to administer more corrosive substances.

Palmitic Acid.—Four rabbits (see protocols appended) received palmitic acid in mineral oil in amounts equivalent to those of the saturated fatty acids. There was in each instance an irritative reaction, entirely non-specific, involving primarily clasmatocytes.

Glycerophosphoric Acid.—The glycerophosphoric acid secured from the phosphatide was given as sodium glycerophosphate in distilled water in amounts equivalent to those which were received by the animals given 80 mg. of phosphatide per dose. The only tissue reaction was a minimal response of leucocytes and clasmatocytes. Likewise, when glycerol C.P. was given in mineral oil, only the finding of phagocytized oil droplets in the cells of otherwise unstimulated milk spots gave evidence of the series of injections the rabbits had received.

Glucose and the *sugar acid* have not been tested on the cells of the omentum. Thus, the biological evidence for the non-specificity of the subfractions, other than the saturated fatty acid, from the phosphatide, confirms the original view that the principle active in the production of tubercular tissue is quantitatively contained in the fatty acid.

Stearic Acid.—An isomer of stearic acid, which Dr. Anderson has termed "tuberculo-stearic acid," was isolated as one of the constituents of the fatty acid derived from the glyceride fraction. The non-

specific character of the tissue response to this acid has been described in detail in the preceding part of this communication. For comparison stearic acid C.P. was given to five rabbits (see protocols appended). There resulted a minimal reaction, even less than that elicited by its isomer; it reflected a mild response of clasmatocytes, monocytes, and epithelioid cells.

The maximum response of non-specific diffuse connective tissue was elicited by the unsaponifiable partition from the purified wax. The details of this reaction have been included in the preceding section.

A comparative study of the substances reported in this chapter which have not produced epithelioid cells, reveals three as outstanding from the rest in their reactions in the tissues: lecithin, olive oil, and the unsaponifiable substance from the purified wax. Lecithin is the only substance used as a control thus far which has given tissue that could be called tubercular or tubercle-like in its structure. This particular reaction was, however, minimal in amount when compared with the reaction to the phosphatide A-3 from the tubercle bacillus, and consisted of a few localized tubercles in omentum, mesentery, and wall of the intestine in areas otherwise not much thickened. The olive oil gave rise to an extreme reaction of large, foreign body giant cells. The unsaponifiable substance produced fully as much new tissue as the phosphatide A-3, but none of it was in the form of tubercles; instead there was a marked new growth of general connective tissue cells. These three reactions were sufficiently distinctive for the nature of each irritant to be identified from a study of the tissues.

In all the other controls, the calling of leucocytes into the tissues and a stimulation of clasmatocytes to phagocytic action have been the common reactions; there have also been some increase in monocytes, a minimal formation of epithelioid cells, the production of giant cells, plasma cells, and, occasionally, a local increase in lymphocytes. None of these reactions, however, have been sufficiently distinctive to enable one to identify from the appearance of the sections the reagent used.

PROTOCOLS OF RABBITS

Controls. All Injections Intraperitoneal

R 709. 12 doses of 10 cc. aq. dest.—No increase in milk spots, slight vascular dilatation in omentum. Clasmatocytes and serosal cells predominant. No in-

crease of monocytes. Foci of primitive cells and lymphocytes. Bone marrow showed decrease in fat content.

R 730. 12 doses of 10 cc. normal saline.—Omentum showed no change in the gross. Supravitally, findings similar to those in R 709.

R 247. 12 doses of 0.25 mg. H-37 tubercle bacilli (inactivated at 60°C. for 1 hr.) in 10 cc. normal saline. Total of 3 mg. in 14 days.—Omentum showed some increase in milk spots with clasmatocytes and lymphocytes predominating. Very few epithelioid and Langhans' giant cells seen. One small typical tubercle found in fixed tissue.

R 248. 16 doses of from 0.25 to 3 mg. H-37 tubercle bacilli (inactivated at 60°C. for 1 hr.) in 10 cc. normal saline. Total of 7 mg. in 19 days.—Omentum definitely stimulated. Many clasmatocytes, plasma cells, and a marked infiltration of lymphocytes. Occasional area of beginning necrosis with leucocytic infiltration. Tubercles were small and all surrounded by a dense thick wall of lymphocytes. Typical tuberculous granulation tissue in limited amount.

R 243. 15 doses of from 80 to 122 mg. lecithin in 10 cc. aq. dest. Total of 1,242 mg. in 17 days.—Omentum appeared normal in the gross except for one pink nodule and some vascular dilatation. Increased phagocytosis by clasmatocytes. Reaction of perivascular adventitial cells. The nodule was a clump of epithelioid cells like a tubercle with a few Langhans' giant cells.

R 244. 15 doses as in R 243.—Omentum showed moderate thickening with some pink bands. Predominant reaction was of clasmatocytes highly phagocytic, with many large vacuoles. Phagocytized material all stained with neutral red and afterward with Sudan III. Occasional epithelioid cell seen in supravital films and the pink bands were clumps of epithelioid cells with a few epithelioid giant cells.

R 245. 15 doses of lecithin plus killed tubercle bacilli in 10 cc. aq. dest. Total of 1,242 mg. lecithin and 3.75 mg. tubercle bacilli in 17 days.—Omentum maintained its normal delicacy but with decided increase in milk bodies. Supravitally there were many epithelioid cells and clasmatocytes, with serosal cells prominent.

R 246. 15 doses. Total of 1,242 mg. lecithin and 4 mg. tubercle bacilli in 17 days.—Omentum showed some vascular dilatation, with milk spots only moderately apparent. Predominant reaction was of clasmatocytes and lymphocytes with occasional epithelioid and Langhans' giant cells.

R 338. 15 doses of 5 cc. olive oil.—Few adhesions and great quantity of fat in peritoneal cavity. Omentum thickened, with many clasmatocytes, fibroblasts, and very large foreign body giant cells. Epithelioid cells and epithelioid giant cells few and scattered.

R 341. 15 doses of 5 cc. olive oil.—Massive fat in peritoneal cavity. Omentum only moderately stimulated, much of it remaining delicate and thin. All connective tissue cells filled with oil droplets. Very few epithelioid cells. Predominant reaction was of large foreign body giant cells scattered singly in the areolar tissue with very little cellular reaction otherwise. Very few epithelioid giant cells.

R 665. 11 doses of 5 cc. olive oil.—Serosa of cecum markedly irritated; omentum thickened, fibrous, and inflamed. Much fat, free and adherent, and an excess of opaque oily fluid in peritoneal cavity. Predominating cell in fluid and omentum was the monocyte, with many single and multinucleated epithelioid cells. There were more large foreign body giant cells than small epithelioid giant cells. Reaction mixed and very complex. Several small abscesses with infiltration of neutrophils.

R 800. 2 doses of 5 cc. olive oil plus 2 cc. 1 per cent Niagara blue intravenously.—Predominantly a clasmatocytic reaction as proven by accompanying intravital stain. Few neutrophils. Very little reaction in general.

R 829. 12 doses of 5 cc. olive oil plus 1 per cent trypan blue intravenously. Total 26 cc. trypan blue in 13 days.—More general cellular reaction in omentum than in other rabbits with olive oil. Monocytes, epithelioid cells, and small epithelioid giant cells were predominant. Clasmatocytes and foreign body giant cells complicated the picture. Trypan blue in finely divided state formed the rosette in the epithelioid series and appeared in larger aggregations in clasmtoacytes and foreign body giant cells.

R 398. 1 dose of oleic acid 0.1 cc.—Many neutrophilic leucocytes. Some phagocytized by clasmatocytes. A few young monocytes seen. One small abscess. Otherwise normal omentum.

R 400. 2 doses of 0.1 cc. oleic acid.—Adhesions between cecum and peritoneal wall, sharply localized. Omentum normal in the gross. Many neutrophils and clasmatocytes in certain areas.

R 402. 3 doses of 0.1 cc. oleic acid.—Adhesions between cecum and parietal peritoneum. Serosal cells irritated. Most of neutrophils are in clasmatocytes. Young monocytes but no epithelioid cells seen.

R 435. 1 dose of 1 cc. oleic acid.—Excess of peritoneal fluid with white granular exudate over cecum and liver. Omentum showed vascular dilatation and many neutrophils and clasmatocytes. Few young monocytes present. Multiple abscesses.

R 444. 1 dose of 1 cc. oleic acid.—Some increase of milk spots in omentum but otherwise content of peritoneal cavity normal in gross. Many actively phagocytic clasmatocytes and young monocytes. Few changes of primitive cells. No epithelioid cells seen.

R 1025. 12 doses of 0.1 cc. oleic acid in 0.5 cc. mineral oil.—Adhesion of cecum and loop of small intestine to peritoneal wall. Much fibrinous exudate. Omentum moderately thickened. Clasmatocytes the predominant reaction. Neutrophils and fibroblasts, but very few monocytes. No epithelioid cells. Few foreign body giant cells.

R 459. 1 dose of 1 cc. mineral oil.—Omentum normal. Milk spots composed of clasmatocytes. Few young monocytes, and plasma cells. No epithelioid cells. No increase of neutrophils.

R 511. 3 doses of 0.5 cc. mineral oil.—Omentum normal. Branched clasmatocytes not stimulated.

R 503. 12 doses of 0.5 cc. mineral oil daily.—Small amount of opaque peritoneal fluid containing many neutrophils, monocytes, serosal cells, and occasional epithelioid cells and clasmatocytes. Much fat in omentum; otherwise normal. Clasmatocytes contained some phagocytized red and white blood cells. Few monocytes, occasional epithelioid cell. Many neutrophils. Many vacuolated mononuclears. Very occasional vacuolated giant cell. Plasma and primitive cells.

R 621. 12 doses of 0.5 cc. mineral oil daily.—Omentum entirely normal in the gross. Many foci of plasma cells and lymphocytes. Some vacuolated mono- and multinuclear cells. Occasional neutrophils. Some monocytes and few epithelioids, and foreign body giant cells.

R 622. 12 doses of 0.5 cc. mineral oil daily.—Peritoneal cavity normal and very few cells in the opaque fluid. Omentum only showed evidence of reaction. Many monocytes and clasmatocytes, few epithelioid and epithelioid giant cells. Many foreign body giant cells, some with vacuoles. Frequent foci of lymphocytes.

R 660. 10 doses of 0.5 cc. mineral oil daily.—Peritoneal cavity contained a small amount of opaque fluid. Cell count 7,000 per c.mm. Neutrophils 13 per cent; lymphocytes 19 per cent; monocytes 66 per cent; clasmatocytes 1 per cent; unclassified 1 per cent. Omentum showed stimulation only in the portion dorsal to the greater curvature of the stomach. Areas between milk spots practically unstimulated. Many of cells of milk spots contained oil droplets obscuring cytoplasmic structures. Typical monocytes and clasmatocytes abundant. No neutrophils or lymphocytes. Epithelioid giant cells and foreign body giant cells very rare.

R 661. 12 doses of 0.5 cc. mineral oil daily.—Small amount of opaque fluid in the peritoneal cavity contained 26,800 cells per c.mm. Neutrophils 21 per cent; lymphocytes 30 per cent; monocytes 33 per cent; clasmatocytes 6 per cent; unclassified 10 per cent. Omentum showed some increase of milk spots and a few pink, thickened areas. Some plasma cells, monocytes, occasional epithelioid cell and epithelioid giant cell. Many lymphocytes. Predominant reaction was of clasmatocytes, fibroblasts, and foreign body giant cells. See Plate 6, Fig. 19.

R 662. 12 doses of 0.5 cc. mineral oil every 48 hours.—Some increase of fluid in the peritoneal cavity. Neutrophils 36 per cent; lymphocytes 22 per cent; monocytes 39 per cent; clasmatocytes 2 per cent; serosal cells 1 per cent. Omentum showed only a moderate increase of milk spots, composed either of clasmatocytes or monocytes. Interspaces unstimulated. Both epithelioid and foreign body giant cells few in number. Occasional epithelioid cell, monocytes frequent.

R 663. 12 doses of 0.5 cc. mineral oil every 48 hours.—Cellular peritoneal fluid. Neutrophils 30 per cent; lymphocytes 19 per cent; monocytes 46 per cent; clasmatocytes 2 per cent; serosal cells 2 per cent. Considerable white exudate, and omentum thickened. Branched stimulated clasmatocytes. Small foci of monocytes with some multinucleated rosette cells. Also foreign body giant cells. Many scattered neutrophils and occasional abscesses.

R 1030. 12 doses of 0.5 cc. mineral oil.—Considerable free oil in peritoneal cavity. Omentum showed a very slight reaction of milk spots. Clasmatocytes with phagocytized neutrophils predominated. Some milk spots were composed of monocytes. Both eosinophils and neutrophils present.

R 1060. 12 doses of 0.5 cc. H-4 purified mineral oil.—Small amount of free opaque oily fluid in peritoneal cavity. Omentum showed some increase in milk spots, with undifferentiated cells and some monocytes. Very rare giant cell of either type found. Predominating cell, clasmatocyte. Minimal reaction on whole.

R 554. 2 doses of 16 mg. palmitic acid in 0.5 cc. mineral oil.—Most of cells of omentum unstimulated. Few neutrophils, clasmatocytes with phagocytized white blood cells. Usual monocytes.

R 555. 3 doses of 16 mg. palmitic acid in 0.5 cc. mineral oil.—Some irritation of serosa of cecum. Omentum delicate but with increased milk spots, made up chiefly of young monocytes. Occasional epithelioid cell. Very little stimulation otherwise.

R 1026. 12 doses of 16 mg. palmitic acid in 0.5 cc. mineral oil.—Fibrinous exudate on cecum. Omentum thickened; increased vascularity with general clasmatocyte stimulation. Few monocytes.

R 1028. 12 doses of 16 mg. palmitic acid in 0.5 cc. mineral oil.—Cecum covered with fibrinous exudate, also diaphragm. Omentum thickened with overwhelming clasmatocytic reaction. Few monocytes and lymphocytes. Some indifferent connective tissue cells.

R 553. 2 doses of 16 mg. stearic acid in 0.5 cc. mineral oil.—Omentum and peritoneal cavity entirely normal in gross and under the microscope.

R 559. 3 doses of 16 mg. stearic acid in 0.5 cc. mineral oil.—Omentum, otherwise normal, showed prominent milk spots, made up of clasmatocytes with leucocytes phagocytized. Few foci of monocytes. Interspaces entirely unstimulated.

R 1057. 12 doses of 5 mg. stearic acid in 0.5 cc. mineral oil.—Only a very slight reaction in peritoneal cavity. Omentum contained very small milk spots, composed of monocytes. Clasmatocytes branched and unstimulated in interspaces. Occasional epithelioid cell, rarely with multiple nuclei.

R 1058. 12 doses of 5 mg. stearic acid in 0.5 cc. mineral oil.—Some free fibrinous plaques in peritoneal cavity. Omentum showed diffuse thickening. Few neutrophils and lymphocytes. Many young monocytes, few epithelioids, occasional epithelioid giant cell. Some increase in fibroblasts and clasmatocytes.

R 1059. 12 doses of 20 mg. stearic acid in 0.5 cc. mineral oil.—Adhesion between cecum and parietal peritoneum. Very little evidence of reaction in omentum. Free and phagocytized neutrophils in clasmatocytes. Dominant cell was the monocyte, with occasional epithelioid cell and epithelioid giant cell. Some large foreign body giant cells.

R 638. 2 doses of 16 mg. glycerol in 0.5 cc. mineral oil.—Omentum entirely unstimulated in gross, and microscopically the usual clasmatocytes with few monocytes were found.

R 639. 3 doses of 16 mg. glycerol in 0.5 cc. mineral oil.—Omentum had accentuated milk spots, but the interspaces were entirely normal. Clasmatocytes were in the interspaces and comprised some of the milk spots; monocytes were found in others; clasmatocytes contained oil droplets.

R 640. 12 doses of 16 mg. glycerol in 0.5 cc. mineral oil.—Omentum showed no gross changes. Milk spots contained both monocytes and undifferentiated cells. All the oil droplets were limited to the cells of the milk spots. Clasmatocytes remained unstimulated in the interspaces. Few plasma cells. One foreign body giant cell seen with large vacuoles.

R 832. 7 doses of 4 mg. sodium glycerophosphate (from H-37 A-3) in 10 cc. aq. dest.—Peritoneal cavity and viscera normal. Omentum normal in the gross, showed microscopically only increased neutrophils, both within and without clasmatocytes. Usual monocytes. No giant cells.

R 833. 12 doses of 4 mg. sodium glycerophosphate (from H-37 A-3) in 10 cc. aq. dest.—Very slight increase in number and size of milk spots in the omentum. No thickening. Milk spots composed of phagocytic clasmatocytes, with considerable number of neutrophils. Few monocytes and small lymphocytes. Essentially normal otherwise.

R 433. 1 dose of 0.1 cc. suspension red phosphorus in 1.9 cc. NaCl.—Peritoneal irritation at site of injection. Scraping showed many monocytes. Increased prominence of milk spots only in omentum. Neutrophils and clasmatocytes in moderate number. Foci of young monocytes prominent; some plasma cells.

GENERAL DISCUSSION

The first significant property of chemical partitions derived from bacterial analyses is solubility. On this basis, the fractions from the tubercle bacillus can be divided into two primary groups, the one water-soluble, the other water-insoluble; the former includes the proteins and polysaccharides, the latter the lipoids. The group of the acid-fast organisms is well known to be especially rich in lipoids. It, therefore, has long been recognized as important to determine the biological significance of the tuberculo-lipoids.

The chemical basis for the studies here reported is to be found in the comprehensive analysis made by Dr. R. J. Anderson (9) of the lipoids isolated from one strain of tubercle bacilli, H-37, grown under standard conditions on Long's synthetic media. He has submitted to us all the primary divisions of these lipoids and many of the subfractions. As far as we know, there never before has been the opportunity to test biologically all the different lipoids from one analysis. This chemical study is to be the pattern for the subsequent analyses of

the corresponding material from the other acid-fast organisms, and the biological testing, as here outlined, may thus also serve as a guide for such parallel tissue studies. From the biological standpoint, it has proved of great importance to be able to compare the relative potencies of all the lipoid partitions in the production of epithelioid cells, as well as to determine their power to incite general acute and chronic inflammatory reactions in the connective tissues.

The lipoids from the tubercle bacillus, Strain H-37, have been split by Anderson (9) into four groups, of which two, phosphatide and glycerides, are soluble in alcohol-ether, and two, purified wax and soft wax, in chloroform. In a further grouping, the phosphatide, purified wax, and soft wax are insoluble and the glycerides soluble in acetone. These lipoids and split products from them have been administered to rabbits in the following ways: isolated (fatty acid I), as an emulsion in water (the phosphatide), in solution in water (non-specific substances, oleic, palmitic, and glycerophosphoric acids), or in mineral oil (all the saturated fatty acids, I–V, tuberculo-stearic acid, phthioic acid, the waxes, and the unsaponifiable substance).

In general, the reactions of the normal connective tissue of the rabbit to the lipoids from the tubercle bacillus may be divided into three groups: first, the acute irritative response, involving dilatation of the vessels and an emigration of leucocytes with their subsequent ingestion and disintegration by clasmatocytes or with abscess formation; second, the so-called specific development of epithelioid cells and epithelioid giant cells; and third, the no less significant but more general changes in the connective tissues, including an increase in undifferentiated fixed connective tissue cells, fibroblasts, clasmatocytes, young, free, basophilic mononuclear cells, lymphocytes, plasma cells, and fibrous tissue, together with an active new formation of blood vessels.

The Acute Irritative Response.—The calling of leucocytes from the vessels into the tissues is a general initial response to the introduction of almost any foreign substance into the tissues. It is difficult to find a substance which does not call forth leucocytes, and the reaction follows the injection of living or dead tubercle bacilli and all fractions, whether protein, polysaccharide, or lipoid, as well as all split products of the lipoids so far tested. In general, the leucocytes thus called out

have in these experiments been phagocytized by clasmatocytes more frequently than aggregated into pus. Certainly when the occasional accidental infections have been ruled out, abscess formation has been a relatively minor reaction.

The most interesting point in connection with the acute irritation caused by these different substances has been the readiness with which the tissues have ceased to respond to succeeding injections of certain of the fractions with a fresh emigration of leucocytes. For example, after the administration of phosphatide, only the first dose has called out any number of leucocytes; by the third injection, only the evidence of their destruction by clasmatocytes remains; with the polysaccharide, on the other hand, each injection brings out a fresh emigration of leucocytes. The undiluted fatty acids have a corrosive action, and repeated injections give a marked, sterile peritonitis. When the acids are dissolved in mineral oil, the irritative effects of repeated doses can be reduced to the constant presence in the tissues of some leucocytes, scattered and in small clumps. The tissues after the administration of fatty acids have never been free from leucocytes, as they are with the phosphatide, and occasionally there have been small abscesses. On the basis of the work of Carrel (26), who found that the white blood cells contain growth-producing substances, the question arises as to whether the new formation of connective tissue following the lipoids is in part due to the destruction of leucocytes with the liberation of such stimuli. In this connection, we may say that the destruction of leucocytes is a factor common to all the experiments and thus can hardly account for any differences in cellular reaction. For example, no portion of the tubercle bacillus calls out more leucocytes than the polysaccharides, and yet these fractions cause a very limited formation of new connective tissue. On the other hand, the phosphatide stimulates a massive formation of new tissue, with only a minor and transient emigration of leucocytes. The experiments with the phosphatide, to which the neutrophils become so quickly nonresponsive, make it possible to estimate that the length of time necessary for the complete destruction of leucocytes by clasmatocytes is not more than 2 days.

There is another factor common to the experiments involving repeated doses of the lipoids, namely, that the normal adipose tissue

of the omentum becomes replaced by new connective tissue, so that the possible effects of the breaking up of the lipoids of the tissues themselves must also be taken into account.

Specific Response.—In the production of epithelioid cells and epithelioid giant cells, each of the four primary groups of the lipoids contains certain liquid saturated fatty acids which give a specific reaction comparable in amount to that of the original fraction. Thus it may be said that the four original partitions give a specific reaction proportional to the amount and to the activity of the fatty acids they contain. Arranged in series, the phosphatide and fatty acid I give the maximum reaction, ++++; the soft wax and fatty acid III, +++; the purified wax and fatty acid II, ++; and the active fraction from the glycerides, +. The original primary fraction made up of glycerides has not been tested, but in the fatty acids from this partition it has been shown that the specific activity resides in an optically active fraction, phthioic acid, which in fatty acid IV is present in small amount, enough to give some epithelioid cells, while the optically inactive portion of fatty acid IV, separated out and identified as an isomer of stearic acid, is inert toward epithelioid cells. The specific activity of fatty acid V has been shown to be due to its content of phthioic acid. The amount of specific tissue produced, which we estimate as +, is not directly comparable with that found after the other fatty acids, because of the relatively smaller dosage used. Further chemical studies are necessary before it can be known whether phthioic acid is the only active principle for tubercle formation.

The phosphatide, the waxes, and their fatty acids have caused much production of the specific tissue in the form of closely packed masses of epithelioid cells, making tubercle-like structures, while the fatty acids from the glycerides have brought about no clumping of the epithelioid cells. It remains to be seen whether this is simply a matter of dosage or of the amount of the specific material concentrated in a given place.

Giant cells have been a constant factor in these reactions, but the numbers and the types have varied markedly. With the phosphatide, the Langhans type predominated overwhelmingly. Their occurrence varied from one or two giant cells in a mass of epithelioid cells, through all variations in proportion to epithelioid cells up to pure masses of

giant cells. In analyzing the reactions of all other fractions, it must be remembered that the diluting menstruum, mineral oil, gave rise to a few foreign body giant cells when used alone. Giant cells were in greatest numbers with the purified wax and the fatty acid IV; in these two cases, large foreign body types predominated. It has not been possible to determine fully the reason for the occurrence of giant cells, but the supravital studies of the omentum have often revealed an epithelioid giant cell in the midst of a clump of epithelioid cells, in cases in which it was entirely clear that there was no necrosis in the vicinity. The use of the entire intact membrane rather than a section has made this observation possible and has confirmed the view of Doan, Sabin, and Forkner (7) and of Forkner (8) that the Langhans giant cell is simply a multinuclear epithelioid cell and subject to similar stimuli.

Non-Specific Connective Tissue.—Besides these differences in the number of epithelioid cells and giant cells, there are marked differences in the amount of the so-called non-specific tissue reaction. In general this reaction is extremely complex and involves a new growth of blood vessels with active division of endothelium and adventitia, the new formation of undifferentiated cells, of fibroblasts, clasmatocytes, lymphocytes, and plasma cells. In these experiments the formation of fibrous tissue, except in adhesions, has not been extreme and there has been no marked encapsulation of groups of epithelioid cells, even during the process of resorption lasting up to 5 months. The lipoids that produced the most epithelioid cells, namely, the phosphatide and soft wax, produced the least general connective tissue, while the purified wax and the glycerides produced the most. The purified wax and its derivative, the unsaponifiable fraction, caused the development of connective tissue composed largely of the so-called fixed type, while the phthioic acid produced a tissue rich in free, young, mononuclear cells, monocytes, lymphocytes, and plasma cells. An increase in connective tissue is found around tubercles and as a part of tuberculous granulation tissue in the disease itself, and hence it is significant that such a reaction follows injections of the lipoids, indeed in extreme amounts after certain fractions, namely, the purified wax, the unsaponifiable substance, and the glycerides.

The Nature of "Foreign Body Reaction."—The reactions to the lipoids from the tubercle bacillus have been placed by pathologists

in the group of the so-called foreign body reactions. The experimental studies on the phagocytic activity of connective tissue cells initiated by the work of Metchnikoff and involved in the whole history of the study of living cells with reference to their phagocytic power, call for an attempt to formulate a more definite idea of the meaning of the term "foreign body reaction."

The cells of the connective tissues may be subjected to foreign substances in two ways. Either they may become surrounded by materials which are soluble in the fluids which bathe them, for example, proteins and polysaccharides, which in solution would tend to reach all the cells of the body; or, on the other hand, there may be substances introduced into the tissues which are insoluble, or possibly slowly soluble in the fluids which surround the cells. To this latter group belong the lipoids and certain other substances which, owing to their particulate nature, remain unevenly distributed in the tissues as foreign bodies. Beside the fundamental property of solubility, foreign bodies must be classified according to the size of the molecule or particle; and furthermore, according to whether the cells of the tissues are physiologically adapted to deal with them, when they are engulfed. By general agreement, the term phagocytosis means the engulfing of particles of appreciable size, rather than the diffusion into cells of substances in solution. In supravital staining, a dye like neutral red, which enters the cell in solution, or in fine suspension, merely evidences phagocytosis through the property of the phagocytic cells to secrete this dye and similar vital dyes into the vacuoles of segregation, in which phagocytized particulate matter has been placed.

In these studies, there has been the opportunity in each of the four groups of lipoids to study a physiologically active substance, first in the form of a large molecule, and then in the form of that fraction of the molecule that carries the active principle. This can be illustrated in the case of the phosphatide and fatty acid I, inasmuch as with them the amount of the material and the number of experiments have been more adequate. The ultimate effects of injections of phosphatide and fatty acid I have been the same, namely, a massive production of epithelioid cells and epithelioid giant cells, but the immediate effects have been different. Barring the initial irritation, the overwhelming immediate reaction to one, two, and three doses of phosphatide has

been what we interpret as the phagocytosis of the material by the cells of the milk spots. These phagocytes have been large, round cells, filled with large vacuoles of uneven size but stained with a uniform red color in neutral red. They are the vacuolated cells of Figs. 1 to 3 on Plate 1. This same type of reaction follows the injection of lecithin. On the other hand, the reaction to one, two, and three doses of fatty acid I has been the maturation of typical new monocytes from a more primitive stage, with marked cell division, and by the 4th day signs of their transformation into typical epithelioid cells. During the first 3 days of the injections of the phosphatide, the maturation of characteristic monocytes has also taken place, but in relatively smaller numbers, as compared with the phagocytic cells. By the third dose, however, the actual number of cell divisions in the young, non-phagocytic, mononuclear cells is much greater than in the mature, heavily vacuolated forms. The comparison of the early response to the large molecule (phosphatide) and then to the smaller molecule (fatty acid) suggests that in the case of the phosphatide the cells may at first be occupied with a process of chemical disintegration which with the fatty acid has already been accomplished in the test-tube. The simpler substance, the fatty acid, appears to be concerned physiologically with the extremely complex processes included in the term maturation, which involve rapidly repeated cell division during a period in which the cytoplasm changes from an immature to a mature state. There is considerable evidence that the epithelioid cell, with its large rosette of tiny bodies, is derived from the monocyte, with its small rosette of larger bodies; but certain crucial points for the proof of this view are still lacking. We have some means for detecting the general signs of phagocytosis in the vacuoles of segregation, which stain supravitally, and can identify certain phagocytized materials in these vacuoles, such as carbon, iron, dyes in particulate form, and whole cells, but we have no means of discriminating other chemical waste products and their stages of disintegration. We do not know the nature or content of the vacuoles that make the rosette of the normal monocyte, nor whether this rosette is a preformed organ of the cell or appears only after the cell has begun to function by taking in material. In the monocyte and in the epithelioid cell, the bodies of the rosette react with a uniform color to neutral red, which we assume

indicates that they are at about the same hydrogen ion concentration. It seems clear from these experiments that epithelioid cells can be made to appear by the introduction into the tissues of certain fatty acids and larger molecules from the tubercle bacillus. If this results from the phagocytosis of these substances by monocytes, not by an indirect chemical influence on the cells, then the monocyte and epithelioid cell must have the power of breaking this material into very fine particles, but, after it has been thus finely divided, of changing it only very slowly. In other words, the cell is not physiologically adapted to any such rapid disintegration of this particular phagocytized material as has been demonstrated for the clasmatocyte, in which whole leucocytes have been reduced to a granular débris in 2 days. Epithelioid cells have been found apparently unchanged 5 months after their production by chemical means. Thus, while it may well be that the formation of epithelioid cells after the lipoids belongs in the general group of so-called foreign body reactions, the processes involved in the reaction are more complex and less well understood than the phagocytosis of materials that can be more readily identified and followed in the macrophages of the tissues.

These studies have shown that in tuberculosis, the production of the essential lesion, the tubercle, or rather of its essential unit, the epithelioid cell, is due to lipoids from the bacillus, and specifically to certain hitherto unknown fatty acids of high molecular weight. It is, of course, well recognized that the epithelioid cell is not the unique reaction of the disease tuberculosis, and hence the demonstration that certain specific lipoids are the effective agents in this disease, important as it is to the pathology of tuberculosis, does not demonstrate that tuberculo-lipoids are the sole agent in the production of epithelioid cells and epithelioid giant cells. In many pathological processes other than tuberculosis, epithelioid cells as such are found; further, they are readily produced, as is shown in our studies with other foreign bodies, especially lecithin, injected into the tissues, and as has been reported in the recent publications of Cunningham and his co-workers (27). It remains to be seen whether there is any common chemical factor in the production of epithelioid cells, or whether the common factor may not be material of varied chemical composition, which the cell can disperse into fine particles, but cannot change or digest with

speed. Because of the differing properties of the phagocytic cells, the power of the monocyte-epithelioid strain to disperse finely yet but slowly change phagocytized material and that of the clasmatocyte or macrophage to digest material quickly, we find convenient the concept that there are two strains of phagocytic cells in the connective tissues differing in their physiological activities. The other alternative is the assumption of a single strain of cells which react differently as the environment is varied.

Beside the opportunity to study the larger and the smaller molecules of a physiologically active substance, the fatty acids from the glycerides have provided an opportunity to study biologically active principles in increasing purity and especially to give an example of an optically active substance which is more biologically active than a similar substance optically inert.

Throughout these experiments, any extreme, acute, inflammatory reaction like that resulting from an accidental infection, or from an extreme reaction to a diluting menstruum, such as olive oil, has tended to obscure the specific formation of tubercular tissue. Caseation has been found only after the injections of the phosphatide.

In spite of great activity in the production of new connective tissue, the lipoids have not induced any skin sensitivity. Intracutaneous skin tests with Old Tuberculin, the protein, lipoid, and polysaccharide fractions have been consistently negative, as well as tests of the conjunctiva in rabbits. Subsequent inoculation with living tubercle bacilli has resulted in no allergic phenomena.

CONCLUSIONS

1. The lipoid fractions from the tubercle bacillus contain maturation factors for monocytes, epithelioid cells, and epithelioid giant cells.

2. The most important component of the lipoids for biological investigation is the phosphatide A-3, since this produces the most massive reaction toward epithelioid cells and epithelioid giant cells, and also because it is the only partition of the lipoids which acts as an antigen.

3. The stimulus to the formation of tubercles resides in certain fatty acids of high molecular weight found in tubercle bacilli. These fatty acids are present in the four major partitions of the lipoids and account in each instance for their specific activity. The most potent fatty

acid in the production of tubercles is that derived from the phosphatide. The purified, optically active phthioic acid obtained from the glyceride fraction, in small dosage produces epithelioid cells, but more non-specific connective tissue; the optically inactive tuberculo-stearic acid is relatively inert.

4. The specific tubercular tissue resulting from the intraperitoneal injection of the phosphatide from the tubercle bacillus undergoes resorption. Two mechanisms in its disappearance similar to those operating in the disease have been seen: caseation in which masses of degenerating epithelioid cells become infiltrated with leucocytes; phagocytosis of the cellular débris by clasmatocytes, without caseation.

5. Beside the specific reaction of the lipoids, these also produce a marked growth of non-specific connective tissue cells, without, however, any reaction toward fibrous tissue in the acute stages. All the subfractions from the lipoids are irritating when injected into the peritoneal cavity, calling leucocytes into the tissues and stimulating clasmatocytic activity. The unsaponifiable substance from the purified wax is particularly active in producing an extreme general reaction of connective tissue cells.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Section of omentum of Rabbit R 821, after three intraperitoneal injections of 80 mg. of phosphatide A-3 from H-37 in 10 cc. distilled water. The figure shows vacuolated phagocytic cells of a milk spot; one containing an engulfed leucocyte. The two cells with dense cytoplasm on the left border are possibly a monocyte and an epithelioid cell. Hematoxylin and eosin. × 1,000.

Fig. 2. Section of omentum of Rabbit R 821,—to show the range of free cells, vacuolated phagocytic cells, some containing leucocytes, free leucocytes, lymphocytes, and possibly monocytes. Hematoxylin and cosin. × 1,000.

Fig. 3. Section of omentum of Rabbit R 821,—to show cell division of the monocytes. Hematoxylin and eosin. × 1,000.

Fig. 4. Film of living omentum of Rabbit R 664, after eleven intraperitoneal injections of 80 mg. of phosphatide A-3 in 10 cc. distilled water,—to show milk spot consisting of epithelioid cells supravitally stained in neutral red. The material stained red in the living state appears dark in the photograph. Supravital neutral red. × 95.

Plate 2

Fig. 5. Section of omentum, Rabbit R 478, after seven intraperitoneal injections of 10 mg. of phosphatide A-3 from H-37 in 2 cc. distilled water,—to show a small group of typical epithelioid cells with dense cytoplasm. One in division. Hematoxylin and eosin. × 790.

Fig. 6. Section of wall of cecum of Rabbit R 319, after twelve intraperitoneal injections of 80 mg. of phosphatide A-3 from H-37,—to show a group of atypical epithelioid cells with foamy cytoplasm. Hematoxylin and eosin. × 790.

PLATE 3

FIG. 7. Film of living omentum. Edge of a milk spot of the omentum of Rabbit R 664, after eleven intraperitoneal injections of 80 mg. of phosphatide A-3 in 10 cc. distilled water,—to show the supravital reaction of coarse neutral red granules in epithelioid cells and epithelioid giant cells. Supravital neutral red. × 1,050.

FIG. 8. Film of living omentum of Rabbit R 664, after eleven intraperitoneal injections of 80 mg. of phosphatide A-3,—to show two small epithelioid giant cells with fine neutral red bodies. The refractile bodies represent serosal cells; clasmatocytes are seen indistinctly at a different level. Supravital neutral red. × 1,050.

FIG. 9. Section of omentum of Rabbit R 158, after twelve intraperitoneal injections of 80 mg. of phosphatide A-3 in 10 cc. distilled water,—to show a massive formation of epithelioid cells, with a few epithelioid giant cells and a mass of lymphocytes. Hematoxylin and eosin. × 150.

FIG. 10. Section of omentum of Rabbit R 153, after twelve intraperitoneal injections of 80 mg. of phosphatide A-3 in 10 cc. distilled water,—to show masses of epithelioid cells with many epithelioid giant cells, moderately vascularized. Hematoxylin and eosin. × 63.

PLATE 4

FIG. 11. Section of omentum of Rabbit R 153, after twelve intraperitoneal injections of 80 mg. of phosphatide A-3 in 10 cc. distilled water,—to show small groups of epithelioid cells and tubercular granulation tissue. There are many epithelioid giant cells. Hematoxylin and eosin. × 132.

FIG. 12. Section of omentum of Rabbit R 153, after twelve intraperitoneal injections of 80 mg. of phosphatide A-3 in 10 cc. distilled water,—to show tubercular granulation tissue. Hematoxylin and eosin. × 176.

FIG. 13. Section of a thoracic lymph node along the internal mammary vein of Rabbit R 319, after twelve intraperitoneal injections of 80 mg. of phosphatide A-3 in 10 cc. distilled water,—to show a lesion made wholly of epithelioid giant cells. Hematoxylin and eosin. × 397.

FIG. 14. Section of a thoracic lymph node of Rabbit R 319, after twelve intraperitoneal injections of 80 mg. of phosphatide A-3 in 10 cc. distilled water,—to show a small focus of early caseation. Hematoxylin and eosin. × 397.

PLATE 5

FIG. 15. Section of omentum from Rabbit R 502, which had received twelve intraperitoneal injections of 16 mg. of fatty acid I in 0.5 cc. mineral oil. It shows a tubercle-like structure surrounded by lymphocytes. Hematoxylin and eosin. × 195.

FIG. 16. Section of omentum from Rabbit R 613, which had received twelve intraperitoneal injections of 16 mg. of fatty acid I in 0.5 cc. mineral oil. It shows a multiple, tubercle-like structure with giant cells and a capsule of lymphocytes. Hematoxylin and eosin. × 200.

FIG. 17. Section of omentum from Rabbit R 613. It shows tubercular granulation tissue and both types of giant cells. Hematoxylin and eosin. × 200.

FIG. 18. Section of omentum from Rabbit R 614, which had received twelve intraperitoneal doses of 16 mg. of fatty acid I in 0.5 cc. mineral oil. It shows tubercle-like structures and tubercular granulation tissue. Hematoxylin and eosin. × 200.

PLATE 6

FIG. 19. Film of omentum, supravitally stained in neutral red, from Rabbit R 661, which had received twelve intraperitoneal doses of 0.5 cc. mineral oil. Supravital neutral red. × 180.

FIG. 20. Section of omentum of Rabbit R 615, which had received twelve intraperitoneal injections of 16 mg. of fatty acid II in 0.5 cc. mineral oil. It shows tiny tubercle-like structures, an epithelioid giant cell, and tubercular granulation tissue. Hematoxylin and eosin. × 200.

FIG. 21. Section of omentum of Rabbit R 615. It shows two epithelioid giant cells with a small, tubercle-like mass of monocytes and epithelioid cells between them. Hematoxylin and eosin. × 960.

FIG. 22. Section of omentum of Rabbit R 615,—to show an epithelioid giant cell with central area of necrosis and one phagocytized leucocyte. There are typical monocytes. Hematoxylin and eosin. × 1,000.

FIG. 23. Section of omentum of Rabbit R 615,—to show a group of monocytes, one of them in division. Hematoxylin and eosin. × 1,000.

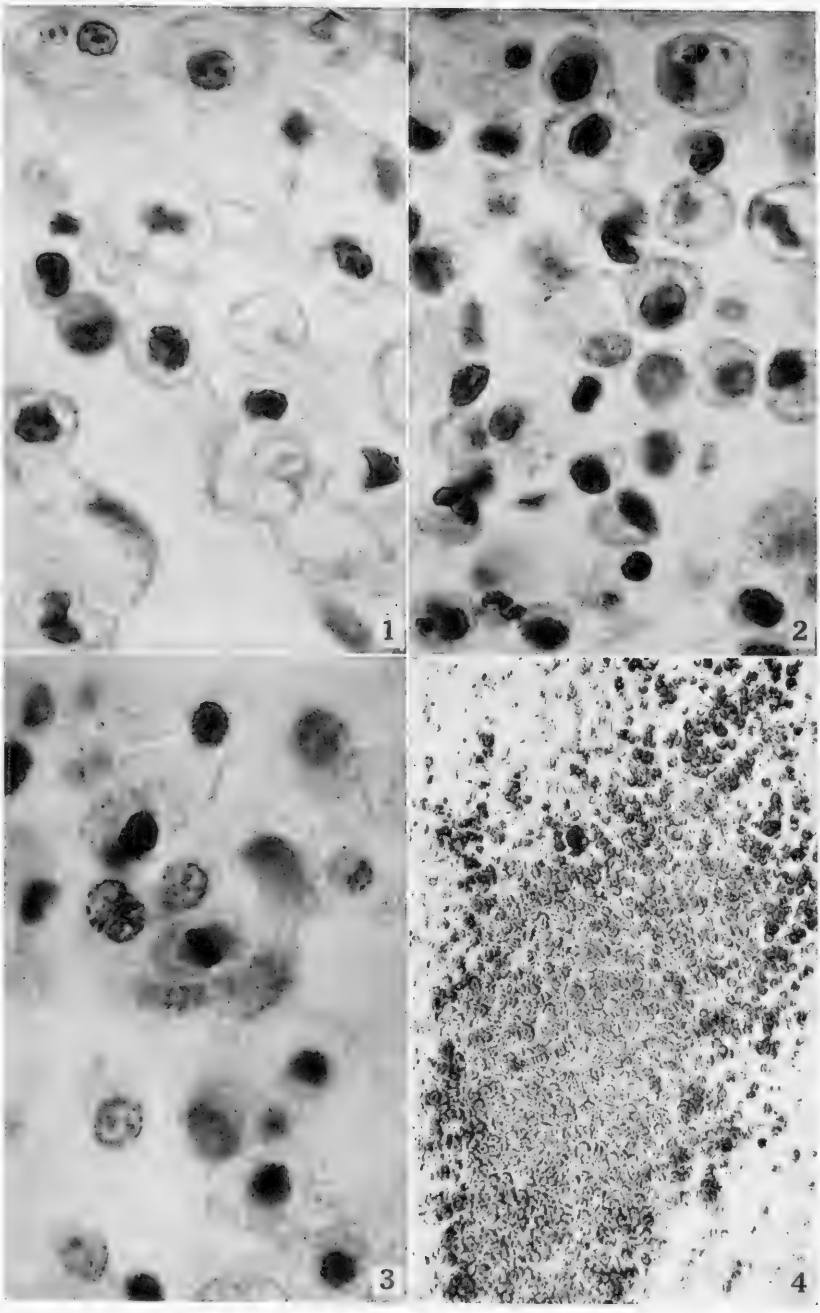
PLATE 7

FIG. 24. Section of the increased serosal membrane over the diaphragm of Rabbit R 948, which had received twelve intraperitoneal injections of 16 mg. of soft wax in 0.5 cc. mineral oil. It shows the extreme dilatation and congestion of the small veins and some tubercle-like masses of epithelioid cells diffusely infiltrated with leucocytes. Hematoxylin and eosin. × 200.

FIG. 25. Section of omentum of Rabbit R 618, which had received twelve intraperitoneal injections of 16 mg. of fatty acid III in 0.5 cc. mineral oil. It shows multiple tubercle-like structures with masses of lymphocytes. Hematoxylin and eosin. × 200.

FIG. 26. Section of the mesentery near the mesenteric lymph node of Rabbit R 618. It shows a tubercle-like structure and a giant cell of mixed type which has phagocytized a small cell and shows vacuoles. Hematoxylin and eosin. × 200.

FIG. 27. Section of omentum of Rabbit R 921, which had received twelve intraperitoneal injections of 20 mg. of fatty acid V in 0.5 cc. mineral oil. It shows diffuse epithelioid cells without any arrangement in tubercle-like structures. They are in active division. Hematoxylin and eosin. × 1,000.



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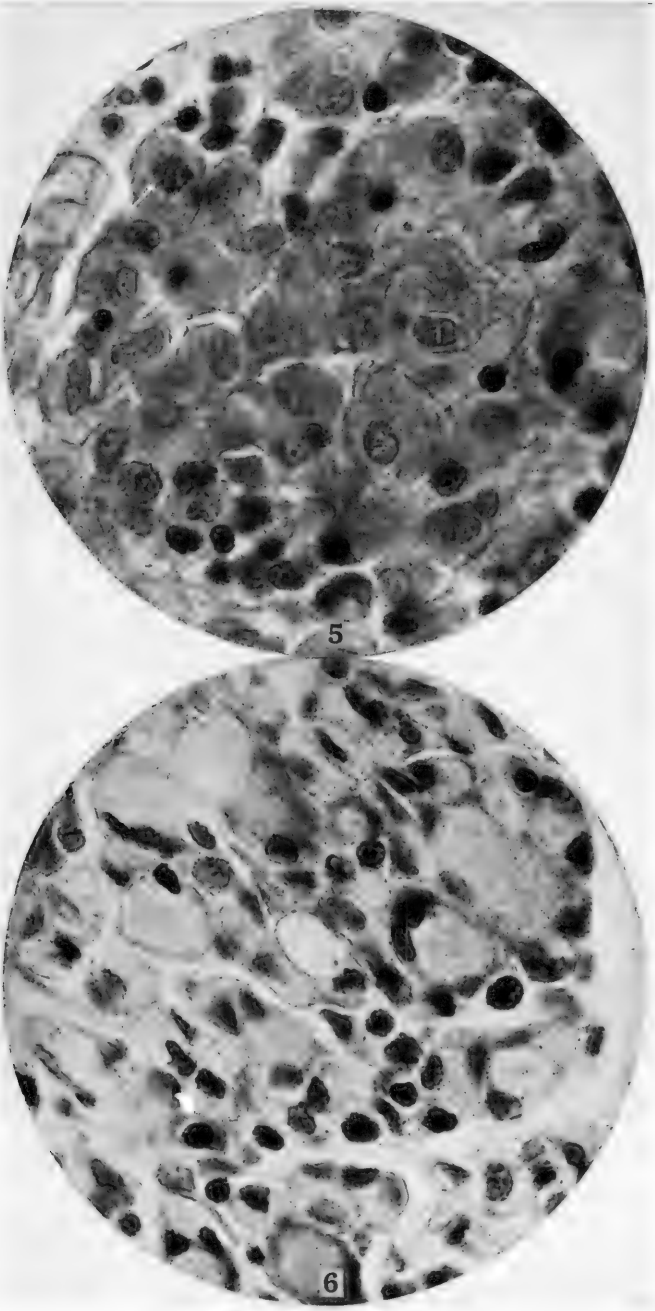

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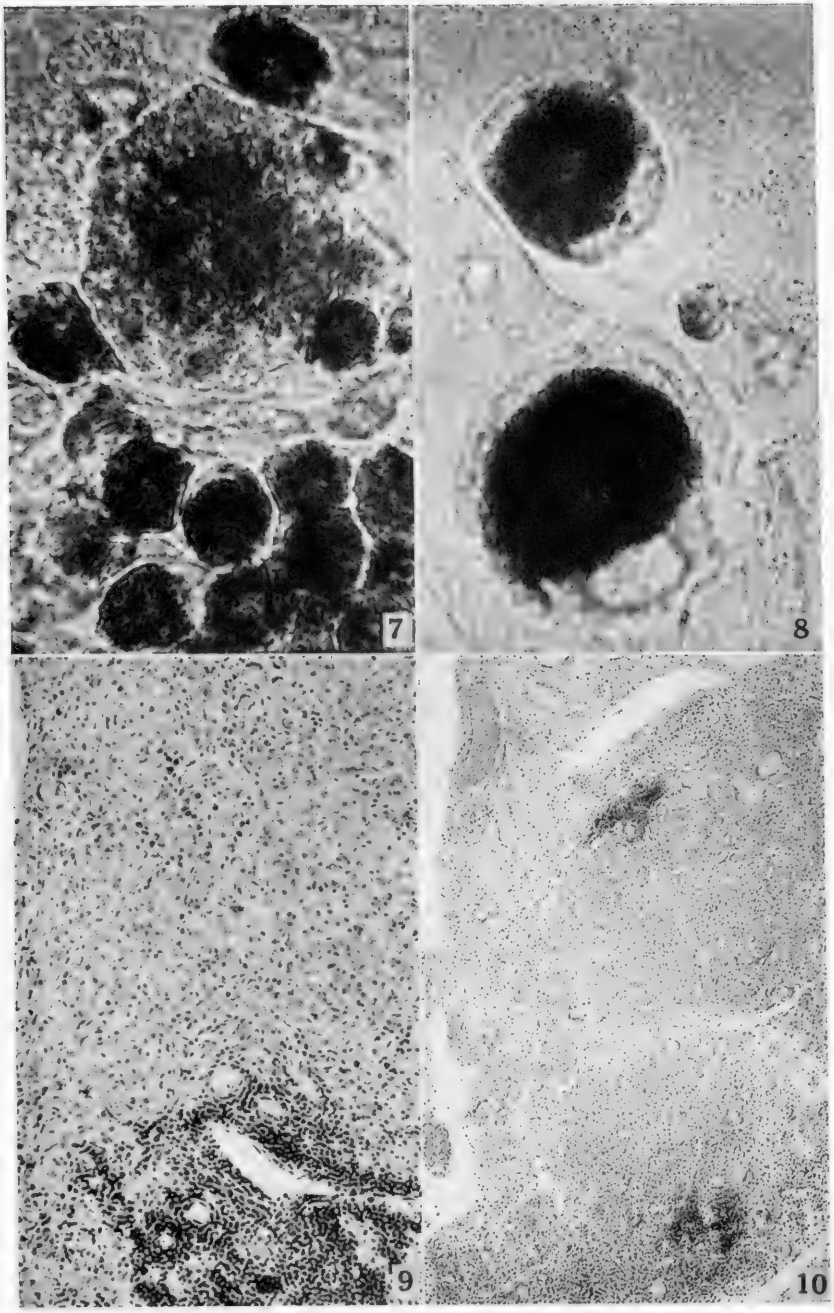

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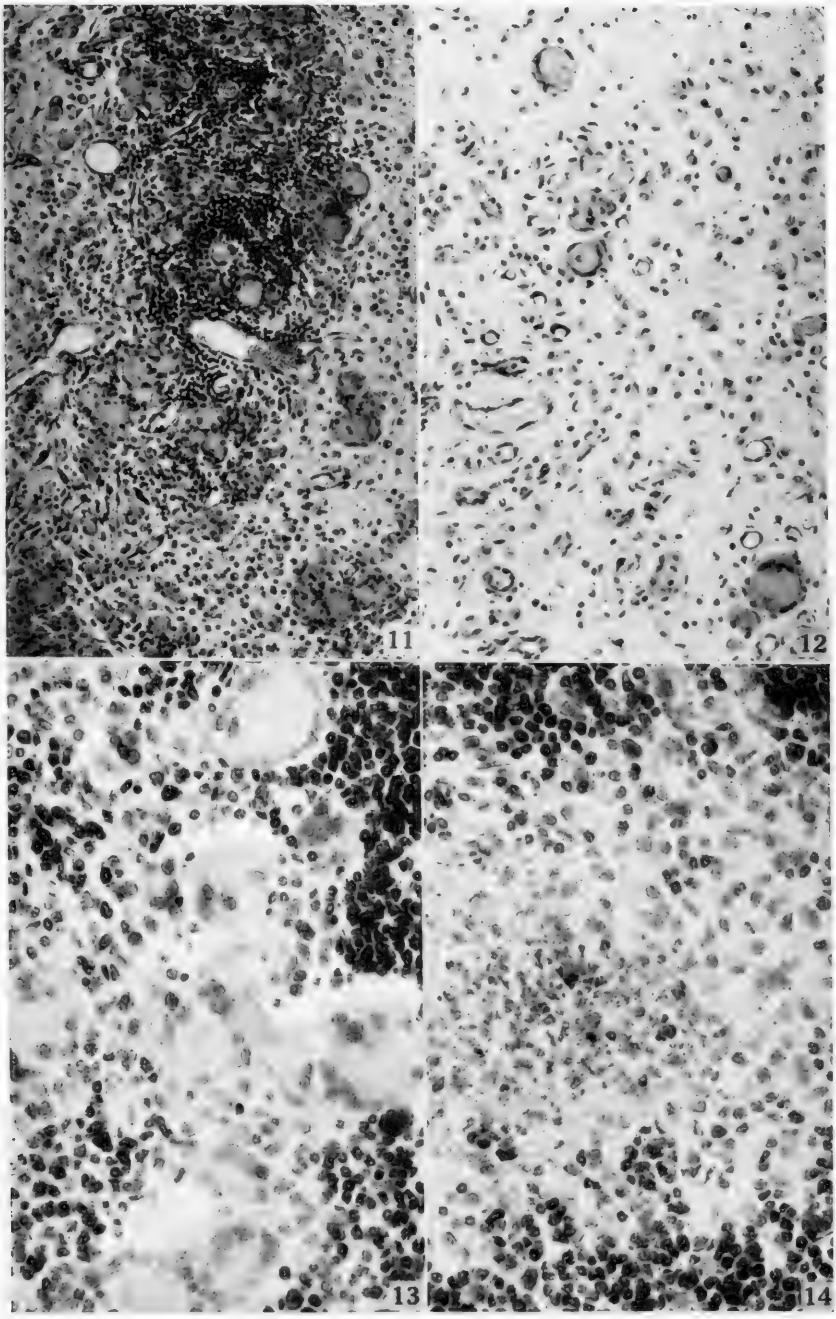

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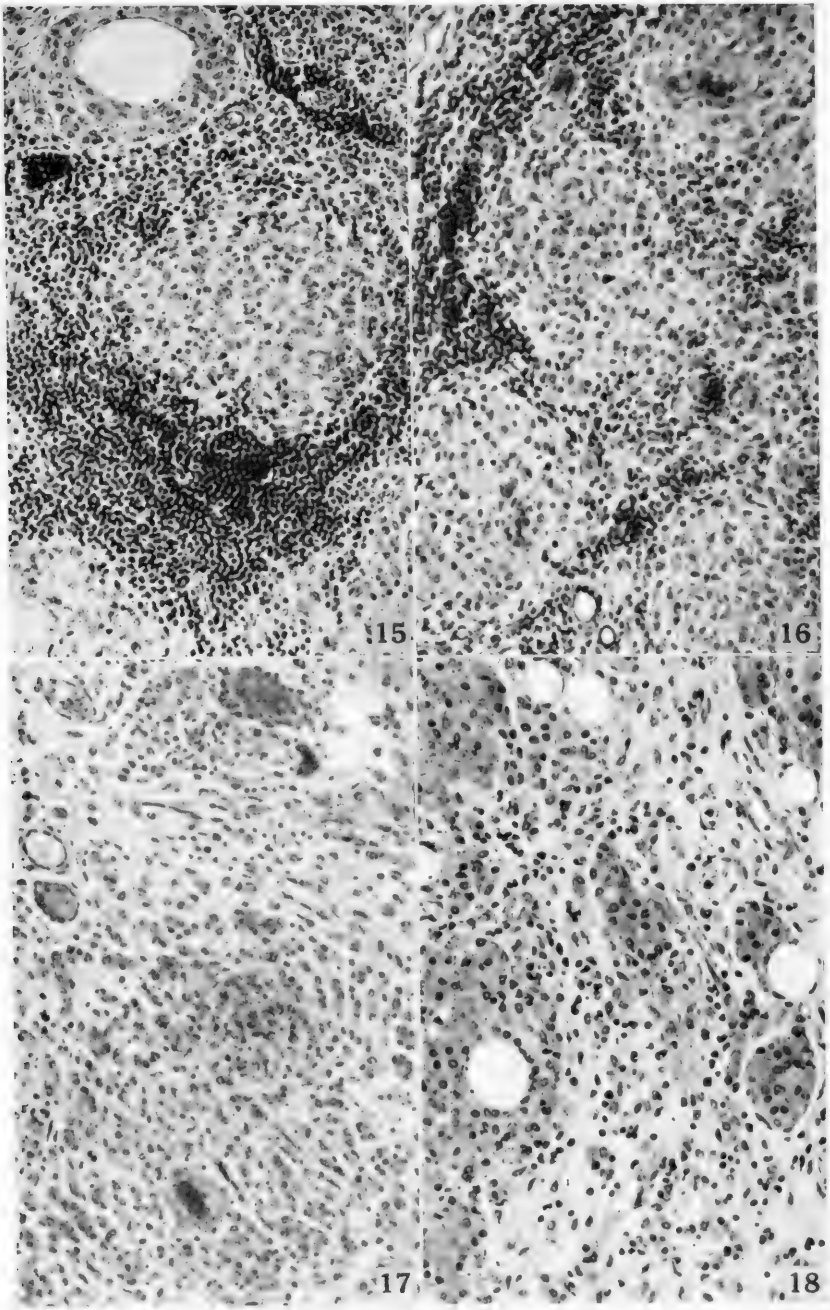

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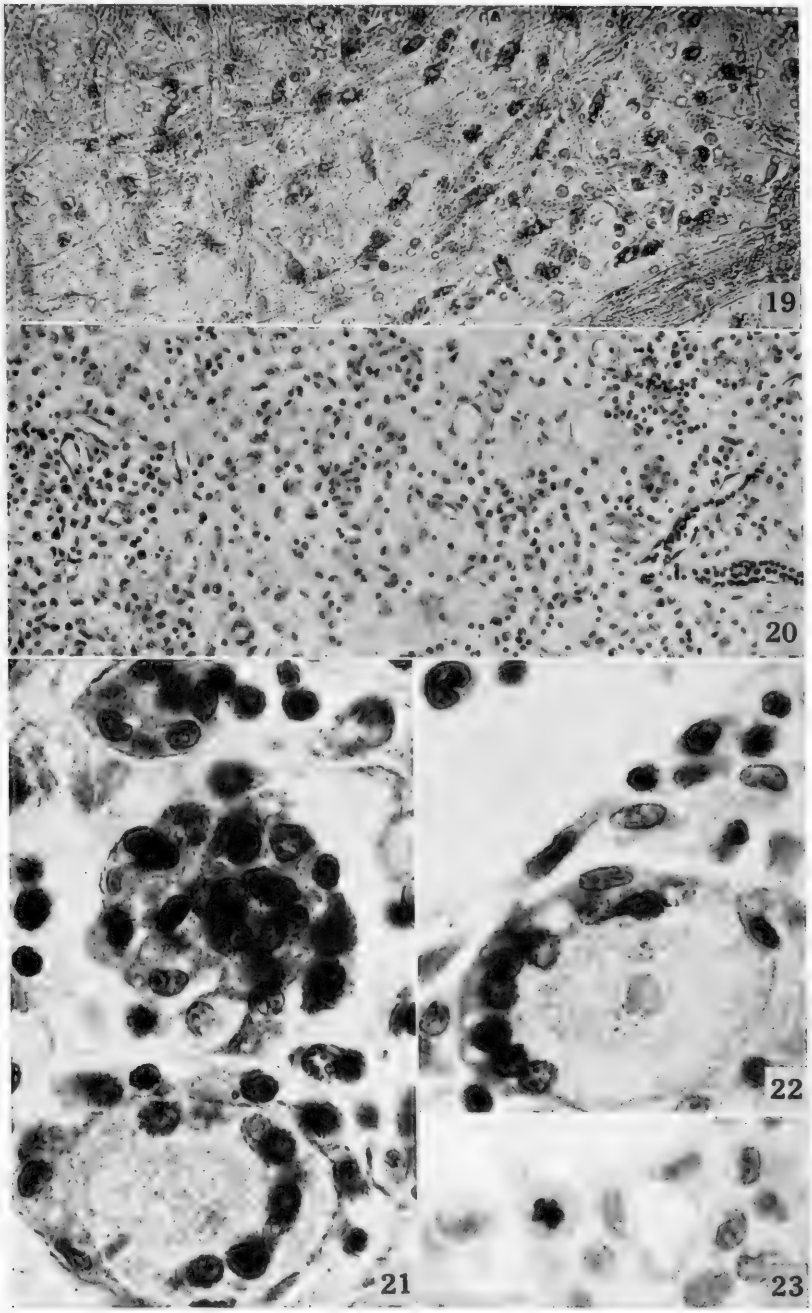

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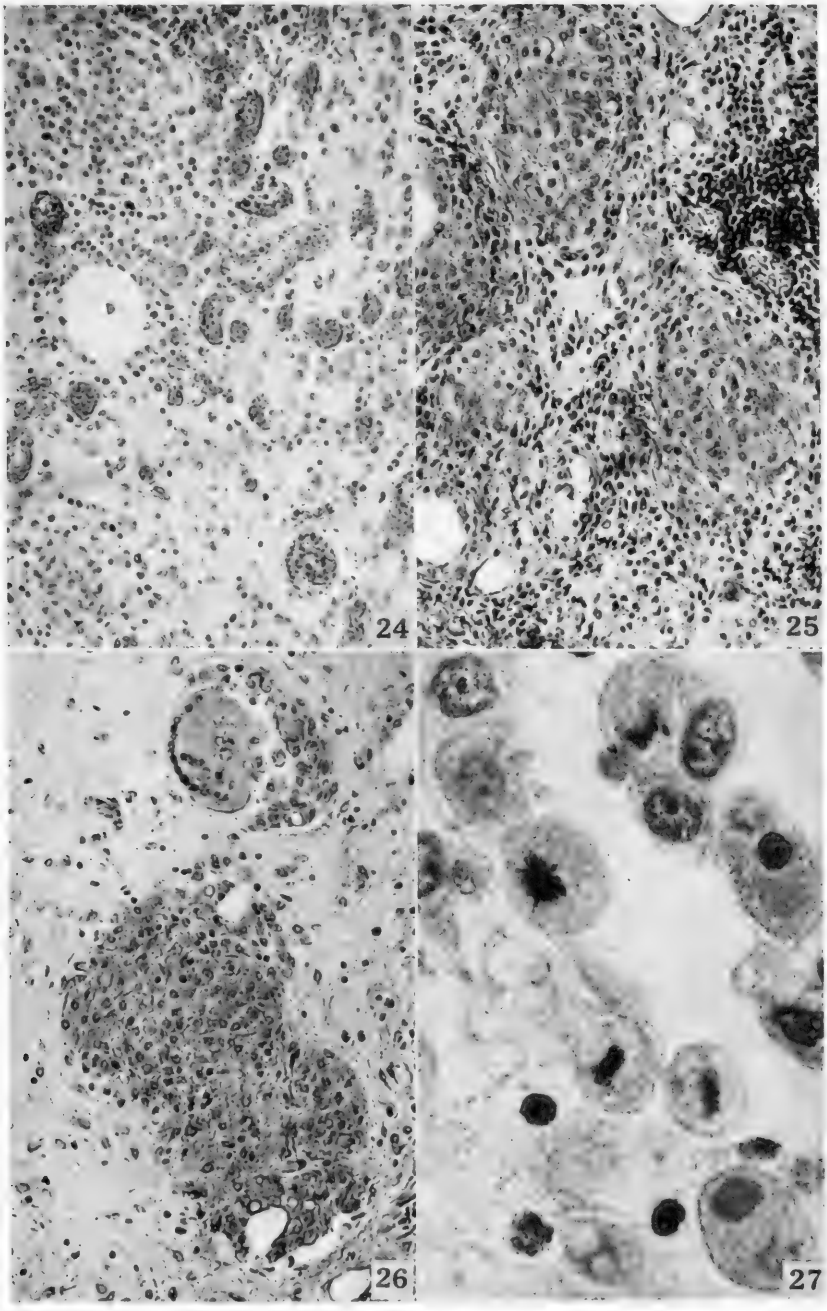

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CHAPTER II

REACTION OF THE CONNECTIVE TISSUES OF THE NORMAL RABBIT TO A WATER-SOLUBLE PROTEIN AND A POLYSACCHARIDE FROM THE TUBERCLE BACILLUS, STRAIN H-37: SPONTANEOUS PSEUDO-TUBERCULOSIS ASPERGILLINA AS A COMPLICATION IN FRACTION TESTING

By CHARLES A. DOAN, M.D., FLORENCE R. SABIN, M.D., and CLAUDE E. FORKNER, M.D.

Plate 8

Reaction to the Protein

In the first chapter (1), we have analyzed the effects on the connective tissue elements of the rabbit, of the intraperitoneal injection of the lipoids from the tubercle bacillus, Strain H-37. With certain of these lipoids, it was possible to reproduce the lesions of tuberculosis in the tissues, including the development of epithelioid cells and epithelioid giant cells in tubercle-like masses and the formation of tubercular granulation tissue. It next became of significance to see if the proteins and polysaccharides of the bacillus, introduced into the tissues by the same route, contributed toward the specific lesions of the disease. We have been able to show that the proteins and polysaccharides thus far tested, when introduced intraperitoneally, do not give rise to tubercular tissue. These substances are soluble in water and hence it is not probable that they remain localized in the subserosal tissues as do the lipoids. Nevertheless it can be demonstrated that they are not inert in their passage through a structure like the omentum.

The intravenous and the intradermal routes are also important for testing the biological reactions to the fractions. From the standpoint of the study of the disease, tuberculosis, the testing of the effects of the proteins and polysaccharides in the tuberculous animal is more important than in the normal.

All these studies are being made under the plan for cooperative research already outlined (1). The analysis of the media in which tubercle bacilli have been grown is being made by Dr. Esmond R. Long and Dr. Florence Seibert at the University of Chicago, who are developing chemical and biological studies of the tuberculoproteins (2–5). From the media, Dr. Seibert has isolated water-soluble protein in crystalline form; in its basic chemical structure it seems to be the same protein as that obtained from the bacilli by Johnson, Coghill, and Renfrew, and designated 304. Dr. Seibert has shown that this protein is probably the active principle in tuberculin; that it kills sensitized guinea pigs in appropriate doses. Her observations indicate that this protein molecule is extremely labile, readily undergoing changes that affect solubility and biological properties. In its natural form it is soluble and crystallizable; but with slight manipulation the original solubility is so changed that instead of dissolving at pH 5.4 in distilled water it dissolves at a pH of 7.0, and the protein will still give a skin reaction but will not crystallize. A still further manipulation renders it insoluble, except in an alkaline medium, under which conditions the biological properties are lost. Long (6) has made further significant biological studies with this protein. By perfusing the renal vessels of tuberculous swine, he has produced an intra-glomerular tuberculin reaction resulting in a so-called glomerular nephritis.

Under the direction of Dr. Treat B. Johnson, a comprehensive analysis of the chemical constituents composing the tubercle bacillus is being made at Yale University (7–9). After the lipoids had been extracted from the bacilli by Dr. R. J. Anderson, the proteins and other products from the residue have been isolated by Drs. Johnson, Coghill, and Renfrew, who have also followed the increasing development of polysaccharides in the media as the bacilli grow and die in the cultures (10). These polysaccharides have not yet been tested biologically.

The original water-soluble proteins submitted to us for testing were prepared by Drs. Johnson, Coghill, and Brown (7–9), and were designated 304 and 903. The differences in the preparation of these two specimens have been outlined by Dr. Johnson as follows:

"Both 304 and 903 are protein fractions extracted by cold water from unautoclaved tubercle bacilli after defatting for 2 weeks with ether at ordinary temperature.

"The only difference in technic employed is that 304 was precipitated from the cold, sterile, aqueous extract after the latter had been centrifuged in an ordinary high speed cup centrifuge only. No. 903, on the other hand, was precipitated from a corresponding sterile, aqueous extract which had been run through the high speed supercentrifuge twice before precipitation of the protein.

"That 304 was a less pure product chemically was indicated by the results obtained when we analyzed the products for nitrogen.

304	Nitrogen content	14.40 per cent
903	Nitrogen content	15.50 per cent

"I would conclude from our chemical data that 903 probably contains a higher concentration of the pure protein than 304. Also it is very probable that 903 is slightly more soluble, due to its higher purity."

These proteins were tested in 1927 by Sabin and Doan (11), by the intravenous route in normal rabbits.

It was found that though they were far less toxic in the normal than in the tuberculous animal, nevertheless they were not inert in the former when given in sufficient doses. Both specimens were soluble in distilled water and were given from a saturated solution. Protein 903 was the more toxic, the lethal dose being smaller. The effects noted were anorexia, loss of weight, temperatures ranging to 108°F., a rise of leucocytes in the peripheral blood, an anemia, extensive diffuse hemorrhages in organs and connective tissues, marked proliferation of clasmatocytes in the interstices of the septa of the lungs, causing a so-called interstitial pneumonia, and, in sufficient doses, death. These studies indicate that certain toxic phenomena which characterize the infection of tuberculosis can be produced in a normal animal by intravenous injection of the active protein in sufficient dosage.

New samples of water-soluble proteins from the bacilli prepared by Drs. Johnson and Renfrew, and designated 304-A and 304-B, have been received more recently. These proteins were prepared from the residue after the extraction of the alcohol-ether-soluble substance by Dr. Anderson. For protein 304-A, the original bacteria were not autoclaved before the alcohol-ether extraction, while for 304-B they were inactivated. Both of these samples proved to be much less soluble in water than either of the original proteins, 304 and 903. In making up the saturated solution, there was a large insoluble residue; correlated with this was a decreased toxicity (10).

A series of eleven doses of the saturated solution, 10 cc. each, were injected intravenously into four rabbits (R 311 and R 318 receiving 304-A, and R 313 and R 316, 304-B). The animals showed no loss in weight; there was fever to 106°F. and 107°F.; in all but one there was a leucocytosis of 20,000 white cells, the neutrophilic leucocytes being from 10,000 to 12,000 cells. All the rabbits developed a slight anemia with a fall of approximately 1,000,000 red cells, correlated with a drop in hemoglobin of from 1.69 to 3.04 gm. per 100 cc. (Newcomer). There was a tendency toward recovery from the anemia during the injections. At the suggestion of Dr. Johnson we then put a greater amount of protein into solution by first dissolving it in N/10 sodium hydroxide and neutralizing with hydrochloric acid. In this way 5 mg. of the protein were dissolved in 5 cc. of fluid and at this

stronger concentration were given in eight daily doses to Rabbits R 311 and R 313. During these injections there was a second fall in red cells and hemoglobin, of nearly the same magnitude as the first. As in all previous experiments with chemical substances from the tubercle bacillus, intracutaneous tests with Old Tuberculin, the protein, the phosphatide, and the polysaccharide were consistently negative. When given intravenously in six antigenic doses of 25 mg. each to two rabbits, R 702 and R 703, this relatively insoluble protein 304 produced no demonstrable precipitins in the blood serum.

The four animals receiving the daily injections of protein were killed while in excellent condition, and showed in general the same effects, which were not more marked in the rabbits that had received the extra five doses. There were minute hemorrhages into the bone marrow in three and in the lungs in the fourth; the one without hemorrhages in the bone marrow (R 316) had the least anemia. There were small patches of thickened septa in the lungs and in three of the four an increase of leucocytes in the spleen, both free and engulfed in clasmatocytes. The hemorrhages might account for the anemia, and the leucocytes in the spleen probably reflected the leucocytoses in the peripheral blood, for they were not found in the animal without leucocytosis.

These experiments with different samples of the proteins have shown that there exist considerable variations in potency. The chemical studies now in progress are being especially planned to assure protein fractions of uniform nature and biological activity. While much more toxic to tuberculous animals, the proteins from the bacilli, when not denatured, are also toxic to normal animals when given intravenously in sufficient dosage. The toxicity has consistently paralleled the solubility in water. In general the effects of the proteins with the possible exception of the hemorrhages have been like those of any foreign protein.

In all the experiments involving the intravenous injections of the proteins, no formation of epithelioid cells, either diffuse or in tubercles, has been detected in any of the tissues. There is no proof, however, that substances injected intravenously actually reach the tissues in an unchanged state. Hence we used the intraperitoneal route.

In experiments comparable to those with the lipoids, eight rabbits were given the water-soluble proteins in intraperitoneal doses, four of them receiving protein 304-B from the bacilli, and four 304-F from the filtrate. These proteins were prepared from the human strain H-37 by the H. K. Mulford Company. They were given in distilled water and enough for 3 days was weighed out, dissolved, and kept in the ice box. We now know that on account of the danger of bacterial

growth in the solution, it would have been better to prepare only enough for each day. Protein 304-B was entirely soluble; doses of 20 mg. in 10 cc. water were given daily to four rabbits (R 950, R 951, R 974, and R 975). Rabbit R 950 died 3 hours after the ninth dose. There was no peritonitis but one segment of the cecum was contracted as if traumatized by the needle at the last injection. We were interested to see if there were any signs of a possible slight protein sensitization. The lungs were markedly red and in sections the veins and capillaries were distended with blood; there was an increase in eosinophilic leucocytes in the capillaries of the lungs and a slight increase in eosinophilic myelocytes in the bone marrow. There were marked changes in spleen and bone marrow, both showing an increase in clasmatocytes loaded with yellow pigment; there were congestion in the omentum and bone marrow and slight hemorrhages in the latter. The other three animals received twelve doses. One of them, R 951, had small abscesses in the omentum and a localized peritonitis in the capsule of the liver. Cultures were not taken but Gram stains of the tissues were negative for microorganisms. Two or three typical epithelioid cells were found in the scrapings from the omentum but none could be found in sections. The omentum in all four rabbits in this group showed free leucocytes, clasmatocytes loaded with yellow pigment, and in all but R 950 considerable development of plasma cells. In all, the changes in the bone marrow were marked, consisting in a complete depletion of the fat, considerable depletion of myeloid elements, congestion, hemorrhage, and yellow pigment, both in endothelium and in free clasmatocytes.

Four rabbits (R 956, R 957, R 976, and R 977) received corresponding intraperitoneal injections of protein 304-F. This protein was not completely soluble in water and gave a turbid suspension; there was a small amount of acid-fast débris, a finding entirely lacking in 304-B. Three of this series showed a peritonitis (R 956, R 957, and R 977); cultures were not taken but Gram stains of the tissues were negative for microorganisms in all of them. In Rabbit R 956 there was a thick exudate in which the differential count showed 70 per cent neutrophilic leucocytes (see Table VIII). All four of this group showed a thickening of the omentum with many free leucocytes, some hemorrhage, an increase in new vessels, clasmatocytes loaded with leucocytes or with yellow pigment, some foci of lymphocytes, and a development of plasma cells. Three of the four showed occasional epithelioid cells in the omentum, never in clumps and never enough to suggest tubercular tissue. The bone marrow in all four showed small hemorrhages and an increase of yellow pigment in cells. In two (R 957 and R 977) there was a shift to the left in myeloid elements corresponding to the draining of leucocytes into the peritoneal cavity; in three there was a considerable depletion of fat and myeloid elements; while in the fourth (R 976) there were areas of hyperplasia and zones where the structure of the marrow was quite disorganized by the hemorrhage.

In the entire group of eight animals the two constant signs were free leucocytes in the omentum and hemorrhage. In the four animals in which the free leucocytes were in small abscesses, and infection was suspected, Gram stains were entirely

negative. The marrow showed a depletion of myeloid elements in seven of the eight instances. All the animals showed small hemorrhages in the bone marrow, frequently in the omentum also. In six of the group there was some increase in lymphocytes in the omentum and foci of plasma cells. In the entire group with the exception of the one animal that died the general effects were slight. The fall in the red cells was slight except in one rabbit, R 976, which lost 2,000,000 red cells and 3.2 gm. hemoglobin. All but two lost from 200 to 500 gm. in weight, but this was not significant considering the short duration of the experiment and the fact

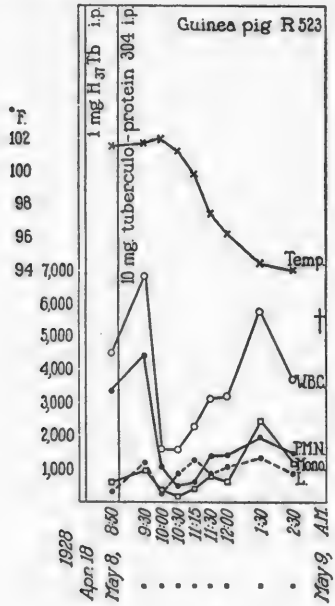

CHART 2

that an adequate base line was not obtained before the injections. The temperature in all reached 103° to 105°F., the upper limits of normal for the rabbit.

The experiment proves hemorrhage to be an effect of the water-soluble tuberculo-protein when administered to normal rabbits. From the standpoint of the general effects of the protein more controls are needed, with cultures and complete bacteriological studies. The experiment demonstrates, however, as a control to the effects of

the lipoids, that the proteins do not produce tubercular tissue by the intraperitoneal route.

In tuberculous guinea pigs (R 523—Chart 2), 10 mg. of the protein 304 given intraperitoneally will cause death within 24 hours, with marked drop in temperature, leucopenia, and showers of monocytes in the peripheral blood.

Reaction to the Polysaccharide

The carbohydrate fraction obtained on analysis of the human tubercle bacillus, Strain H-37, the biological properties of which have been compared with those of the protein and fat fractions, gives strong pentose reactions but its structural formula has not yet been determined. It has been isolated by Dr. Anderson from the small aqueous residue accompanying the original alcohol-ether extract of the bacilli, and it does not include the entire polysaccharide content of the organism. We have studied the action of this particular polysaccharide in normal rabbits, when given by the intravenous and intraperitoneal routes, and in tuberculous guinea pigs on subcutaneous and intraperitoneal injection. This carbohydrate has been designated A-8.

Two rabbits (R 315 and R 317) received 23 daily intravenous injections of 10 mg. of the polysaccharide in 2 cc. sterile glass-distilled water. Daily observations were made of weight, temperature, and blood findings, a preliminary period of 2 weeks serving to establish the normal range in each animal. The rabbits gained just under 1 kilo each during the experiment, with no rise in temperature at any time. The polymorphonuclear leucocytes and monocytes remained within their respective normal limits as did the total white and red blood cell counts and the hemoglobin. The lymphocytes tended in each animal to rise toward the end of the experiment, in one instance from under 2,000 per c.mm. to above 3,000 and in the second from 3,000 to 5,000. Intracutaneous skin tests at two different times, with Old Tuberculin and all three major chemical fractions, were negative and no specific precipitins were demonstrable in the blood serum. The inability of the polysaccharide to elicit demonstrable antibodies as well as its apparently complete inertness when given intravenously was further demonstrated in two rabbits (R 704 and R 705) which received six antigenic doses each of 25 mg. in 5 cc. distilled water over a period of 20 days. No clinical, blood, serological, or skin-sensitive changes were observed. Postmortem studies revealed no gross or microscopic tissue changes that could be detected in any of the rabbits which received the intravenous polysaccharide.

Three rabbits were studied after intraperitoneal injections of the polysaccharide, one (R 454) after three daily injections of 10 mg. in 1 cc. distilled water, and two (R 1022 and R 1023) after twelve injections of 10 mg. in 10 cc. distilled water. With the exception of a transitory fall in total red blood cells in two animals (R 1023 and R 1022), no changes were noted in the number or percentage of any of the cells of the peripheral blood. There was no rise in temperature, and only a slight decrease in weight. It should be noted that the blood counts and the temperatures were taken only once daily.

The tissues of Rabbit R 454 were studied post mortem after three injections for the acute reaction. There was no excess of free fluid in the peritoneal cavity and no evidence of any reaction to the injections except in the omentum. The differential count of the cells in the small amount of peritoneal fluid obtained showed 36 per cent neutrophilic leucocytes, 1 per cent eosinophils, 8 per cent lymphocytes, 43 per cent monocytes, and 12 per cent clasmatocytes (*cf.* Table VIII). The monocytes were all normal and non-phagocytic, while all the clasmatocytes were filled with phagocytized red and white blood cells. The omentum in the gross showed an increase in vascularity and in the size and number of the milk spots. Supravitally the most striking point was the large number of highly phagocytic clasmatocytes, frequently six to eight being found in one oil immersion field, each containing from ten to twelve disintegrating neutrophilic leucocytes and some red blood cells. There were also free granulocytes, some increase in young fibroblasts, and some small clumps of monocytes, but no epithelioid cells. In section, the existence of increased vasodilatation and the predominance of the leucocytic and clasmatocytic reaction were readily confirmed. Much of the tissue remained entirely normal. At one point there was an incipient abscess with a large accumulation of still intact polymorphonuclear leucocytes, and in a few other places very much smaller foci. There were some plasma cells and scattered monocytes but no epithelioids. The mesenteric nodes, spleen, liver, kidneys, adrenals, and lungs were entirely normal in the gross and microscopically. The bone marrow was normal in the gross and showed only a slight depletion of the myeloid elements along the margin.

After twelve intraperitoneal injections of the polysaccharide, the findings (R 1022, R 1023) were much the same as those just described. There was no gross evidence of irritation or reaction on the part of any of the tissues or organs in the peritoneal cavity. The differential counts of the free cells averaged 51 per cent neutrophilic leucocytes, 7.5 per cent lymphocytes, 33 per cent monocytes, 3.5 per cent clasmatocytes, 5 per cent serosal cells. Figs. 1 and 2, Plate 8, illustrate the appearance of the clasmatocytes found in the omentum (R 1023), some fusiform and containing finer débris, the majority large and engorged with phagocytized whole blood cells not yet disposed of. Three of these active clasmatocytes are shown in Fig. 1, and two in Fig. 2. An elongated clasmatocyte with fine granules is in the lower right corner of Fig. 1, and free leucocytes are shown in both figures. Aside from this finding, together with that of a moderate number of polymorphonuclear cells, there was no evidence of unusual reaction in milk spots or inter-

spaces. This rabbit (R 1023) showed an occasional monocyte with increased rosette of neutral red bodies and one multinucleated cell; the other animal showed only the normal number and distribution of monocytes (Table VIII).

In brief, the only apparent local reaction in normal rabbits to the polysaccharide from the human tubercle bacillus under the conditions stated consists of an accumulation of neutrophilic leucocytes followed by an activity of the clasmatocytes to phagocytize them.

In contrast to these findings in the normal rabbit, it was first noted by White (12) that not infrequently death ensued promptly after the subcutaneous or intraperitoneal injection of small quantities of the tuberculo-polysaccharide A-8 in tuberculous guinea pigs. The animals showed a progressive fall in blood pressure and body temperature from the time of injection to death. Changes in the adrenals were noted post mortem, but the exact explanation of the phenomenon was not wholly clear.

The polysaccharide, when given in the same quantity, 10 mg., to normal guinea pigs, is apparently as innocuous as in normal rabbits. Either no change in temperature ensues, or there may be a fluctuation within 1°F., and the white blood cells during the course of the 6 or 7 hours following injection present a typical non-specific curve (R 425). The neutrophilic leucocytes after a brief preliminary depression rise as the lymphocytes fall, with a reestablishment of the normal relationships within 24 hours. The reaction is the same in character and time relations whether the subcutaneous or intraperitoneal route is chosen.

In Guinea Pig R 425, after a moderate degree of tuberculosis was induced, an intraperitoneal injection of 10 mg. of polysaccharide was followed by a fall of the neutrophilic leucocytes, lymphocytes, and monocytes and a transitory rise in temperature with a maximum of 1.7°. Apparently, however, there is a point reached in the development of the tuberculous infection beyond which the guinea pig becomes highly susceptible to this sugar. Guinea Pig R 493 received 0.01 mg. of virulent H-37 tubercle bacilli on April 5, 1928. Intracutaneous skin tests made on the twenty-third of the same month, with Old Tuberculin and tuberculo-protein 304, showed +++ and + reactions respectively at 48 hours; phosphatide A-3, tuberculo-polysaccharide, and physiological saline were negative. On April 26, 21 days after the inoculation, 10 mg. of tuberculo-polysaccharide were given intraperitoneally and within 5 hours the animal was dead, the fall in temperature and changes in the blood cells being given on Chart 3. At postmortem the animal was

found to have extensive tuberculosis of liver, spleen, omentum, and pancreas. As compared with Guinea Pig R 425 which survived the polysaccharide, there was quantitatively much more extensive tuberculosis in Guinea Pig R 493.

Inasmuch as this fatal reaction to the tuberculo-polysaccharide is so similar to that already mentioned as following the injection of tuberculo-protein 304 (Chart 2), and since it is known that the polysaccharide contains nitrogen, probably in the form of protein, further

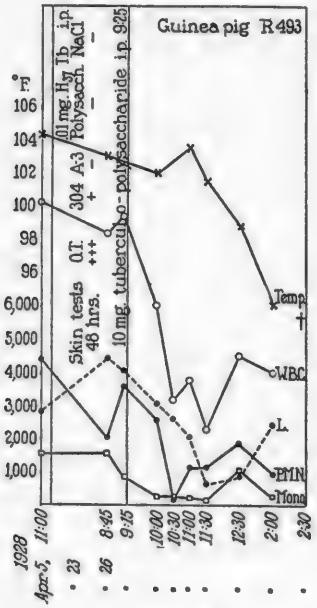

CHART 3

studies are under way to test whether the polysaccharide is toxic *per se* or only as a carrier of protein.

Glucose and pneumococcus type-specific substance III,[1] when given in the same amount to tuberculous guinea pigs as was the tuberculo-protein, caused wholly different effects, the granulocytes tending to

[1] We are deeply indebted to Drs. Avery and Goebel for sufficient of the pneumococcus type-specific polysaccharide for comparative tests with the tuberculo-polysaccharide.

rise within an hour in both instances, the temperature remaining constant after glucose, but rising after the pneumococcus sugar. Chart 4 (Guinea Pig R 498) contrasts two series of observations on the same guinea pig on successive days, the first after pneumococcus polysaccharide and the second after tuberculo-polysaccharide in the same dosage. On the latter occasion it will be noted that after a prolonged precipitous fall in temperature, there was a partial recovery.

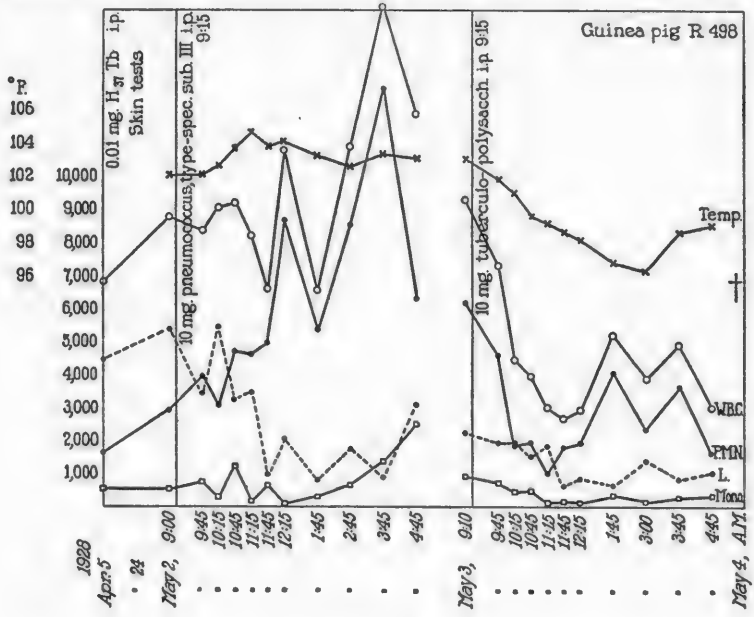

CHART 4

The animal was, however, found dead the following morning. In no instance in which there has been a marked fall in the temperature after the administration of sugar has the animal survived the injection beyond 24 hours. On the other hand, it is our experience that the animal always survives if the temperature remains constant or rises following the injection.

This very marked sensitization of the tuberculous constitution to a specific protein and polysaccharide from the tubercle bacillus seems

to link closely the allergic state of the disease with these soluble substances and tends to show that the phenomenon is not dependent on the lipoid fractions.

Pseudo-Tuberculosis Aspergillina

During the progress of the biological testing of the various fractions from the tubercle bacillus in normal stock rabbits, we have on four occasions encountered in the routine autopsies small, translucent, subpleural nodules in otherwise apparently normal lungs. These solitary nodes from 1 to 2 mm. in diameter were indistinguishable in the gross from small tubercles, and inasmuch as fractions from the tubercle bacillus had been given in each instance, it became of immediate importance to ascertain the nature and cause of the lesions.

Two of the rabbits (R 951 and R 975) had received the protein from the bacilli; one (R 945) had been given the unsaponifiable material from the lipoids, and one (R 613) had received fatty acid I from the phosphatide, all the injections having been by the peritoneal route. Microscopically all four of these lesions were found to be similar in nature, under low power appearing superficially not unlike tubercles (Plate 8, Fig. 3). They were small, intra-alveolar collections of epithelioid cells (Fig. 4) and leucocytes, sharply circumscribed by the walls of the air sacs. The alveolar septa were either not involved or only slightly thickened. Supravital studies had confirmed the presence of epithelioid cells in these areas, and mitotic figures were not uncommonly found in these cells in the sectioned material. Polymorphonuclear leucocytes formed a prominent part of the picture in every instance, infiltrating the lesion and being recognizable in all stages of disintegration, both within and without the mononuclear cells. In none of the lesions could acid-fast bacilli be stained. There was found, however, in each original nodule in the lungs, one or more small, encapsulated, fungus-like structures, two of which are indicated by white arrows in Fig. 4, which were identified as pseudo-tuberculosis aspergillina or pneumomycosis aspergillina, as described by Seifried (13).

The distinguishing characteristic, aside from the presence of the fungus, is the intra-alveolar rather than the intra-septal position of the lesion, making a small pneumonic consolidation, sharply delimited and surrounded by entirely normal lung. It becomes of importance, particularly in the testing of fractions from the tubercle bacillus, to be able to recognize these spontaneous tubercle-like lesions in rabbits.

In this connection, Dr. F. H. Heise of Saranac Lake, New York, has called our attention to a case (No. 5110) of human infection with aspergillina under observation for some time at Trudeau Sanatorium, which showed extensive bilateral pulmonary involvement. This patient gave repeated negative reactions to intracutaneous skin tests with Old Tuberculin in all dilutions up to 0.1 mg., which last gave an inflammatory area 6 x 6 mm. Subcutaneous tuberculin up to 10 mg. was repeated twice, and caused no focal or constitutional reaction that could be determined.

Though the cellular reactions to this fungus are so similar to those resulting from infection with the tubercle bacillus that the lesions are spoken of as pseudo-tuberculosis, the allergic state which accompanies the latter is entirely absent in the former, as would be expected. The pseudo-tubercle of the fungus infection, together with the chemically stimulated tubercular tissue produced by the phosphatide fraction from the tubercle bacillus, serves to emphasize the point which we have made elsewhere (14), that the allergic reactions in tuberculosis are not dependent upon the cellular lesion, the tubercle, *per se*, but are associated with the more soluble specific proteins and possibly with the polysaccharides. These observations support from a somewhat different angle the thesis recently advanced by Rich and McCordock (15), that resistance and allergy in tuberculosis need not be considered as identical.

SUMMARY

From these studies we conclude that the water-soluble protein from the tubercle bacillus, when not denatured, is toxic to normal rabbits, inducing fever and hemorrhage when introduced intravenously, but is not lethal except in massive doses. By the intraperitoneal route it is less toxic, but calls forth a local response of leucocytes and phagocytes without any striking proliferation of new connective tissue. A damage to endothelium is indicated by hemorrhage, chiefly in the bone marrow. Tuberculous guinea pigs succumb rapidly to protein 304 when given intraperitoneally.

The polysaccharide is non-toxic when introduced intravenously in the normal animal; introduced intraperitoneally, on the other hand, it is irritative and each succeeding dose continues to elicit a fresh emigration of leucocytes from the vessels. These leucocytes appear to be

damaged, for they are actively engulfed by clasmatocytes. Guinea pigs with extensive tuberculosis may die soon after subcutaneous or intraperitoneal injections of the polysaccharide.

Aspergillina fungus may produce a pseudo-tuberculous lesion, resembling, both macroscopically and microscopically, the cellular reaction of tuberculosis. The absence of positive skin tests with Old Tuberculin when such lesions are present, as furthermore when tubercular tissue has been produced by chemical stimulation with the tuberculo-phosphatide, emphasizes the necessity for considering tubercle formation as a mechanism apart from allergy in the disease tuberculosis.

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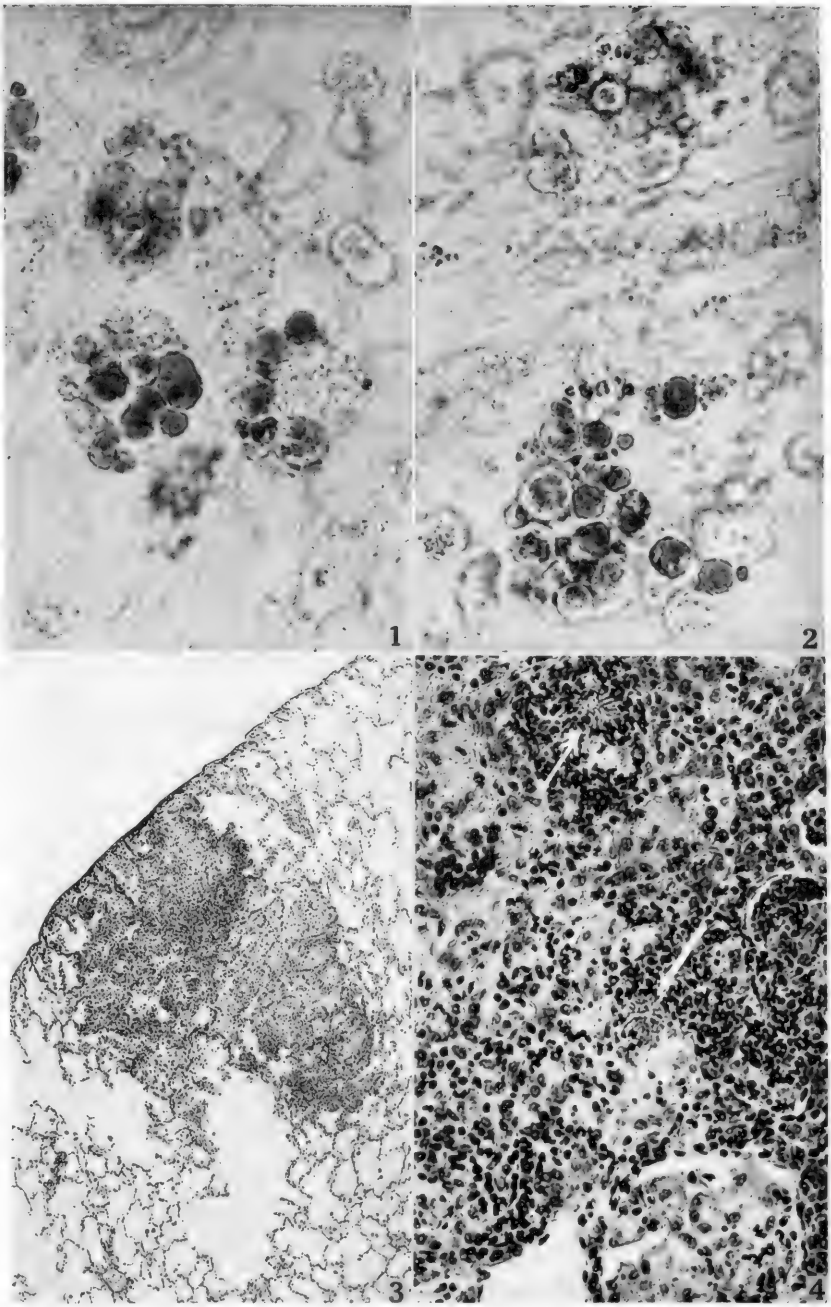
EXPLANATION OF PLATES

PLATE 8

FIG. 1. Film of omentum of Rabbit R 1023 after twelve intraperitoneal injections of 10 mg. of polysaccharide in 10 cc. distilled water. Photographed while the cells were living, stained in vital neutral red. × 1,000. It shows three clasmatocytes filled with leucocytes, free leucocytes, and one unstimulated clasmatocyte in the lower right hand corner. The focus is beneath the serosal cells.

FIG. 2. Film of the same omentum as Fig. 1. In the left hand border is the end of a clasmatocyte containing heavily stained granular débris.



(Doan, Sabin, and Forkner: Water-soluble protein and polysaccharide)

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FIG. 3. Section of the lung of Rabbit R 945 showing a pseudo-tubercle due to the fungus aspergillina. Stained in hematoxylin and eosin. × 56. It shows the intra-alveolar position of the epithelioid cells. The animal had received twelve intraperitoneal injections of the unsaponifiable substance from the purified wax from the tubercle bacilli H-37; the lesion was spontaneous and unrelated to the injections.

FIG. 4. Section of the lung of Rabbit R 975 showing the fungus aspergillina, opposite the two white arrows, and the nature of the intra-alveolar epithelioid cells (lower right hand corner) and free leucocytes. Stained in hematoxylin and eosin. × 258. The animal had received twelve intraperitoneal injections of 20 mg. of protein 304-B from tubercle bacilli H-37; the lesion was spontaneous and unrelated to the injections.

CHAPTER III

THE DERIVATION OF GIANT CELLS WITH ESPECIAL REFERENCE TO THOSE OF TUBERCULOSIS

By CHARLES A. DOAN, M.D., FLORENCE R. SABIN, M.D., and CLAUDE E. FORKNER, M.D.

Plates 9 and 10

INTRODUCTION

The question of the origin and specificity of giant cells found in the connective tissues under various pathological conditions has been for a long time a controversial one. Their frequency in the lesions of tuberculosis has tended to center many of the discussions concerning the nature and characteristics of these cells about this disease. Consequently, when epithelioid cells and giant cells with peripheral nuclei were found in great numbers as a reaction to the injection of lipoids from the tubercle bacillus (1), it became important to study this type of giant cell which is related to a product of the disintegration of the bacilli and to compare it with the giant cells found in tuberculosis and under other abnormal or pathological conditions.

While the name of Langhans has come to be associated only with the type of giant cell with peripheral nuclei, which is so frequently a characteristic of tuberculous lesions, this author described also the type with scattered nuclei. His studies (2) were made with fresh, teased preparations, so he avoided the pitfalls encountered by subsequent workers who used only sections. Recently Medlar (3) has overcome the difficulty of attempting to judge the distribution of nuclei from one section by reconstructions of selected cells from serial sections. He concludes from his own observations, as does Haythorn (4) after a comprehensive review of the literature, that the available histological criteria do not justify a separate classification of the Langhans type of giant cell with peripheral nuclei from the foreign body type with scattered nuclei. While it is true that many giant cells appear in sections to be transitions between these two types, yet the identification of a rosette of vacuoles as the essential characteristic of the cytoplasm of the monocyte (5, 6) and now of the Langhans type of giant cell as well, may give adequate criteria for such a separation and may open the way to an experimental study of the factors producing giant cells.

The cellular structure of a simple tubercle is well known to be a central core of epithelioid cells with one or more Langhans giant cells, the whole frequently surrounded by a capsule of lymphocytes. Our work in tuberculosis has shown, first, that the epithelioid cell, so abundant in the lesions of this disease, is derived from the monocyte by an accentuation of the rosette of tiny vacuoles; and second, that the epithelioid cell in turn gives rise to the Langhans giant cell by an increase in the number of nuclei. Thus we prefer to designate this type of giant cell an *epithelioid*, or *rosette giant cell*, meaning to indicate that it is merely an epithelioid cell with more than one nucleus.

A considerable variety of epithelioid giant cells are shown on Plate 9, in Figs. 1, 4, 6, 7, 9, and 10. All are characterized by a rosette of vacuoles of an approximately uniform size. In general the vacuoles of the epithelioid type tend to be somewhat smaller than those of the monocyte and they may become as fine and dust-like in the rosette of the giant cell as are those of the cell shown in Fig. 7. There are frequently a few larger vacuoles in the periphery of the rosette as in the monocytes; see for example, Figs. 1, 9, and 10. In the cell of Fig. 1, the vacuoles were demonstrated through the reaction to trypan blue given by repeated intraperitoneal injection, while in all the other cells, the rosette was brought out by the supravital reaction to neutral red. No specific stain visualizes the rosette in fixed tissues, except neutral red as retained by special fixation (7–10), but the zone of the rosette stains faintly acidophilic. In the unstained living cell, the rosette appears as finely granular material around a clear area, the centrosphere. This appearance was recognized by Langhans in his original study.

Considering the rosette as the characteristic structure of this type of giant cell, it will be noted that the nuclei are peripheral to the rosette, making as it were an encasing sphere around it. The ratio of the size of the rosette to the diameter of the cell will determine whether the nuclei are at the periphery of the cell itself, as in Fig. 4, or internal to a wide exoplasm, as in Figs. 1 and 6. The difficulties of determining the type of giant cell in sections can be seen readily by comparing the cells of Figs. 2 and 10. In the case of the epithelioid giant cell of Fig. 10, the nuclei could be made to appear unipolar, circumferential, or scattered according to the plane of section.

There is considerable evidence that the multiple nuclei of the epithelioid giant cells come from amitosis of the nucleus of the epithelioid cell. It is in the monoblasts and the early monocytes that the most frequent mitoses are found, when one studies tuberculous lesions and tubercular tissue produced by lipoids from the tubercle bacillus. Young epithelioid cells also divide by mitosis, as can be seen in the example given in Fig. 5 of the first chapter, Plate 2; but we have never found an epithelioid giant cell showing a mitotic figure. Moreover, in cells such as the one of Plate 9, Fig. 7, which is an epithelioid giant cell from the tibial bone marrow of a rabbit (R 1124) dying from tuberculosis, direct evidence of amitosis is shown. One of the nuclei shows a considerable constriction, another had probably just divided with a second constriction in one of the resulting two, while a third nucleus shows another amitotic fission in progress. These nuclear appearances are identical with those seen in the cells of the blood from a case of subacute, myeloblastic leucemia, described some years ago (11), in which amitotic divisions were followed repeatedly from the beginning constriction to complete separation. It seems to us possible that it is the gradual isolation of the centrosome by the increasing size of the rosette of vacuoles which finally inhibits the process of mitosis in the epithelioid cell and thereby necessitates any increase in nuclei by amitosis.

Associated with the concept that the Langhans giant cell is derived from a single epithelioid cell, is the observation that this type of giant cell has a limited range of size and a limited number of nuclei. Rarely have we found a rosette giant cell with more than twenty nuclei; the cell of Fig. 10 has twenty-two. In contrast, the non-rosette type of giant cell frequently has as many as a hundred or more nuclei. The range of size of the Langhans giant cells is well represented on Plate 9, from the epithelioid cell of Fig. 5, which is 8 to 12 microns in diameter, to the cell of Fig. 9, of about 60 microns. There is no such limit to the size of the foreign body type of giant cell and the one of Fig. 2 is by no means the largest we have seen.

The foreign body types, in contrast to the rosette giant cells, are without cytoplasmic pattern. Forkner (12) has demonstrated that this type of giant cell may be formed by the fusion of cells, most commonly by the fusion of monocytes. For this group, the non-rosette

type formed by fusion of cells, we are retaining the usual name, *foreign body giant cell*, without in any way meaning to signify that the epithelioid giant cell is not also formed in response to the phagocytosis of foreign material, since in tuberculosis it does develop in response to lipoids foreign to the body.

Giant Cells in Tuberculosis

With the identification of the Langhans giant cell as a multinucleated epithelioid cell, it becomes clear that, in so far as the epithelioid cell, either in tubercles or diffusely scattered in the tissues, can be considered as characteristic of tuberculosis, its derivative, the epithelioid giant cell, can also be considered characteristic. Wherever the proliferation of new epithelioid cells occurs in abundance, as in tuberculosis, or in the reaction to tuberculo-phosphatide, giant cells with an identical cytoplasmic structure may appear.

If it is true in tuberculosis that the epithelioid giant cells are simply modified epithelioid cells formed in response to lipoids liberated from disintegrating tubercle bacilli, then this type of giant cell should be the first to appear in the early primary reaction to the infection before there is caseation, or extensive necrosis of tissues, or superimposed secondary infection. When any of these latter conditions exist, the reaction is no longer that of the tissues to the tubercle bacillus or its lipoids alone. Corresponding to the more complex nature of the stimuli, cellular responses of increasing variety occur, and giant cells of much larger size and lacking all pattern in nuclei and cytoplasmic structure appear, together with clasmatocytes and neutrophilic leucocytes. These "foreign body" giant cells we have found in tuberculous areas in direct proportion to the amount of tissue destruction and necrosis present. We have studied the relative incidence of the two types of giant cells in acute fulminating tuberculosis as contrasted with their incidence in the more chronic disease, and in the various organs showing lesions.

In the supravital preparations differential counts of the giant cells have been readily made, and subsequently in the fixed tissues a correlation has also been made of the distribution and location of giant cells with reference to tuberculous foci. Of five rabbits (R 849, R 855, R 877, R 856, R 876) dying within 15 to 30 days after intravenous injections of from 0.5 to 0.75 mg. bovine tubercle bacilli,

Strain B-1, three showed only epithelioid giant cells in scrapings from the lungs, and no foreign body types; the fourth showed only one foreign body (non-rosette) giant cell in several preparations studied from the lung, as contrasted with numerous epithelioid giant cells, and only epithelioid giant cells in spleen and marrow surveys; the fifth, dying at 28 days, while showing a great preponderance of rosette giant cells in the lung, still contained frequent non-rosette giant cells as well.

It is interesting to analyze the fixed tissues in the rabbits which showed this difference in giant cell content in the supravital studies. In the first place, the rosette or epithelioid giant cell is usually present in direct association with epithelioid cells, either in the center, or as an integral part, of a tubercle. In the early stages of tubercle formation in the rabbit, the various phases of the rosette cell from monocyte to multinuclear epithelioid cell dominate the reaction (13).

The giant cells which appear first are few, epithelioid in type, relatively small, and not associated with any visible evidence of tissue destruction. This simple condition may not persist long. When degeneration of epithelioid cells starts within a tubercle with infiltration of leucocytes, or when the growing tubercle causes a pressure necrosis on surrounding tissue or organ cells, both conditions involving a lowering of resistance to secondary bacterial invaders, the stimuli become multiple and the reactions correspondingly more complex. It is at this point that non-rosette giant cells appear, probably in response to larger increments of débris to be phagocytized and disposed of, followed by the more chronic infiltration of lymphocytes, again possibly in the attempt to limit the dissemination of the noxious products of bacterial and tissue destruction. Thus the foreign body giant cells appear relatively later in the course of the disease and are found more frequently at the periphery of the tubercle proper, unless extensive caseation has resulted in their wandering into the area of degeneration in a phagocytic capacity. In the fifth rabbit of the series mentioned above, a somewhat more extensive state of caseation in the pulmonary tubercles, as revealed by a survey of the sections, substantiates, in some degree at least, the relationship of this factor to the increased numbers of non-rosette giant cells in this animal.

In this connection it is significant that the lungs as seen in rabbits experimentally infected with tuberculosis contain, especially in the more chronic stages of the disease, more of the foreign body type of giant cells than any of the other tuberculous organs.

For example, in one survey of this kind (R 1124—killed 62 days after 5,000,000 B-1 organisms intravenously), in which giant cells were being particularly noted, of 25 such cells counted in the supravital preparations from the lungs, 7 were of the foreign body or non-rosette type; the peribronchial nodes and tibial bone marrow showed 4 foreign body to 21 epithelioid giant cells; the spleen, 3 foreign body to 22 rosette giant cells; and the popliteal lymph nodes, humeral and femoral bone marrows showed only the epithelioid type of giant cell. The humerus exhibited 25 epithelioid giant cells to 3 megacaryocytes and no foreign body giant cells. In three rabbits (R 734, R 954, R 955), surviving for from 100 to 196 days, preparations from the lungs, in which chronic tuberculous lesions were present, showed both kinds of giant cells, though the epithelioid type predominated; the kidneys, however, showed in the supravital preparations only the epithelioid type. The tendency to destruction of lung tissue and cavitation in chronic pulmonary tuberculosis probably accounts for the frequency with which different types of giant cells have been encountered in this organ in the disease, and for the conclusion so frequently drawn that there is therefore no basis for attributing significance to any particular kind of giant cell in tuberculosis.

The observations given above with the detail limited to a few selected experiments are representative of the studies carried on in this laboratory during the past 5 years upon much larger numbers of animals, under a wide variety of conditions centering about experimental tuberculosis.

It would seem clear that in tuberculosis the Langhans or rosette giant cell can be considered, together with the epithelioid cell, as a direct reaction to the freeing of certain lipoids, notably the phosphatide and certain fatty acids, from disintegrating tubercle bacilli, while in general the reactions in which foreign body giant cells appear are the result of multiple and much more complex stimuli.

Giant Cells after Tuberculo-Phosphatide

In a preceding chapter (14), we have presented evidence showing that certain lipoids from the tubercle bacillus produce the epithelioid cell and tubercle-like structures characteristic of the disease. It is with the giant cells found in response to these lipoid fractions that the interest centers here.

Comparable phosphatide fractions (A-3) from human, bovine, and avian tubercle bacilli have all given the reactions in the tissues characteristic of tuberculosis, that is to say, epithelioid cells distributed diffusely and in clumps resembling tubercles, together with typical

rosette giant cells having peripherally placed nuclei. Repeated supravital surveys of omental spreads and tissue scrapings from numerous animals have shown a marked predominance of rosette giant cells, with but few foreign body types. Indeed, in these experiments the number of the rosette giant cells has far exceeded anything we have ever seen in the disease itself.

For example, the lymph node illustrated in Fig. 13, Plate 4 (14), contained what might be called an almost pure culture of Langhans' giant cells. The extreme number of giant cells found in these reactions with the fractions is probably due to the fact that the amount of tuberculo-lipoid concentrated in the tissues is overwhelmingly greater than that ever liberated in the disease in the same period of time at any one focus.

The cells of Figs. 4 and 5 have been drawn from a supravitally stained spread of the omentum of Rabbit R 684 after intraperitoneal injections of avian phosphatide. It will be seen that the multinucleated cell (Fig. 4) has a massive, unified, central rosette, an accentuation of that seen in the epithelioid cell of Fig. 5, in contrast to the foreign body giant cells of Figs. 2 and 3. The nuclei are far out toward the periphery of the cell, presumably crowded out to this position by the material constituting the rosette. Many of the nuclei have conspicuous nucleoli. This cell is identical with the epithelioid giant cells found after the administration of the other phosphatides, and in tuberculous processes. The epithelioid cell (Fig. 5) is likewise typical of those found in tuberculosis. The fixed sections of the tissues from this animal, as in the others receiving phospholipin, demonstrated the presence and domination of these cell types, in arrangement closely simulating tuberculous tissue.

When the phosphatide was given intraperitoneally in large doses, the reaction in the tissues could not be considered as exclusively due to the phosphatide, because leucocytes were always called from the vessels by the first injection and were phagocytized and destroyed. In a series of experiments to be reported subsequently in greater detail (15), the phosphatide was given intravenously in small antigenic doses and some of the animals used demonstrated the specific reaction to the phosphatide entirely uncomplicated by any other processes. In these animals there were small, scattered foci of epithelioid cells and rosette giant cells, or single epithelioid giant cells in an otherwise entirely normal lung, lymph node, or spleen. This material furnishes the most unequivocal evidence of the direct specific relation of the epithelioid cell and the corresponding giant cell to the phosphatide as stimulus. A single giant cell from the lung of one of these intravenously treated animals is reproduced in Fig. 9, Plate 9. The rabbit (R 844) had received three doses of 10 mg. each of the phosphatide A-3 from human strain H-37 at 5 day intervals. In this giant cell it will be seen that one nucleus lies within the mass of neutral red staining bodies, the remainder of the nuclei being peripherally placed. This is explainable upon the three-dimensional aspect of

the unsectioned spherical cell; the less dense area in the center of the cell indicates the presence of a centrosome about which the neutral red bodies group characteristically in the rosette cells. The presence of ingested carbon particles within the larger vacuoles found so commonly in the phagocytic cells of the lungs helps to identify the organ source of this particular cell and also emphasizes the point that both macrophages or clasmatocytes and rosette cells of the monocyte strain may contain these carbon particles. Nucleoli were very prominent in the majority of the nuclei in this cell. No typical foreign body, non-rosette, giant cells were found.

From the foregoing it will be seen that the phosphatide from the tubercle bacillus introduced into the tissues produces a marked predominance of epithelioid cells and epithelioid giant cells with a minimal number of foreign body giant cells, in this respect closely approximating early non-caseous tuberculosis; while introduced in small doses intravenously, it may give a pure reaction of mono- and multinucleated epithelioid cells.

Giant Cells from the Other Lipoid Fractions from Tubercle Bacilli

The formation of giant cells following the injection of the other lipoid fractions accompanied a more complex reaction in the tissues. Either the formation of epithelioid cells predominated, in which case the rosette giant cell likewise predominated; or there was about an equal distribution between the two types of giant cells and fewer epithelioids; or the non-rosette type was in greater numbers. The chemical analysis of the lipoid is outlined in Table I of the first chapter (14). There it will be seen that certain saturated fatty acids were isolated from each of the four original fractions and carried the specific tendency toward the formation of the epithelioid cell. This was only true when these fatty acids were finely dispersed in mineral oil, for when introduced undiluted, they were corrosive in action.

In the case of the fatty acid from the phosphatide, all the giant cells seen in the fresh preparations were small and of the rosette type; in sections of this material, however, only about half of the giant cells have the typical nuclear arrangement of the Langhans type, while the remainder are like the giant cells shown in the upper part of Fig. 16 and the lower part of Fig. 17 on Plate 5 of the first chapter (14). That is to say, they show nuclei in a dense mass, rather than in the periphery of the cell. From the supravital studies it seems likely that this appearance is due to the plane of section and that these giant cells were for the most part of the rosette type.

In the analysis of the fatty acids, Dr. Anderson fractionated those from the glycerides into two components, tuberculo-stearic acid and phthioic acid.

The tuberculo-stearic acid was followed by the production of the two types of giant cells in about equal numbers and the phthioic acid induced predominantly the rosette types (Fig. 6, Plate 9). Fig. 6 is a cell taken from the omentum (R 918) after intraperitoneal injections of fatty acid IV and illustrates some of the variations which are often found in these cells. Refractive, even sized, unstained vacuoles are scattered in the cytoplasm at the periphery of the cell. The nuclei are not arranged quite so definitely at the periphery of the rosette, three of them being shown underneath and apparently invading to some extent the dense central pattern of neutral red bodies.

Fig. 10 represents one of these rosette giant cells taken from the omentum of Rabbit R 921 which received fatty acid V. It is one of the largest of the epithelioid type we have encountered, containing 22 nuclei, asymmetrical in size and arrangement, surrounding a rosette of neutral red staining bodies smaller in circumference than is commonly found in a cell of this size. The nuclei, which almost all contain prominent single nucleoli, are arranged in single, double, or triple layers closely surrounding the rosette. The periphery of this rosette shows larger vacuoles with and without the stain, a common finding in the rosette cells.

The chemical analyses gave an opportunity to study the reactions of the tissues to a large molecule and then to its component parts. An interesting example of this is the effect of the purified wax and its two components, the unsaponifiable substance and fatty acid II.

Differential counts of the giant cells after administration of the purified wax (in R 937) showed ten epithelioid giant cells to every one of the foreign body type. This tendency was carried directly by fatty acid II which gave rise to the giant cells, on Plate 6. They were especially typical of those seen in the infection. The unsaponifiable material (in R 945), on the other hand, produced as much new tissue as the phosphatide, but this was non-specific in type, and the counts of the giant cells were the reverse of the above, namely, ten to one in favor of the foreign body type. The same relationship of giant cell types was found with the soft wax (in R 948). After both of these materials, the serosal cells were markedly irritated and there were many highly phagocytic clasmatocytes in the omentum.

Oleic and palmitic acids were found in the phosphatide molecule and, as already mentioned, an isomer of stearic acid in the glycerides. Of these three fatty acids, oleic acid proved on biological testing (R 1024, R 1025—twelve injections of 0.1 cc. each) to be the most irritating to the tissues of the peritoneal cavity. Neutrophilic leucocytes, highly phagocytic clasmatocytes, and serosal cell irritation dominated the reaction; few monocytes and no epithelioid cells were found. Only

occasional foreign body giant cells were present. Palmitic acid also proved to be primarily an irritant with an increased activity of the clasmatocytes. Few monocytes were found and only one small rosette multinucleated cell was seen in two animals studied (R 1026, R 1028—twelve injections of 16 mg. each in mineral oil). Stearic acid, however, produced upon the injection of from 60 to 240 mg., in divided doses in mineral oil (R 1057, R 1058, R 1059), a minimal but appreciable reaction of monocytes with occasional epithelioid cells and small epithelioid giant cells. A few neutrophilic leucocytes, a moderate number of clasmatocytes, and an occasional foreign body giant cell completed the cellular detail of the moderate reaction to this fatty acid. There was a marked difference in the size of individual giant cells, those with a central rosette being very much smaller than the ones without this definite cytoplasmic pattern.

Glycerol was also used as a control for these tuberculo-lipoids. When it was given in doses comparable to those of the fatty acids, the reaction (in R 640) was found to be mild, but with epithelioid cells and a few small accompanying rosette giant cells present.

From this study of the tuberculo-lipoids, it is clear that there are certain fractions, notably the phosphatide and certain fatty acids, which give a direct stimulus to the formation of epithelioid giant cells. It is our opinion that this reaction is due to the phagocytosis of the lipoid. On the other hand, there are certain partitions of the lipoid, notably the unsaponifiable material and the soft wax, which produce predominantly foreign body giant cells. As yet we have no crucial experiment, such as the one with small intravenous injections of the phosphatide, to analyze whether these foreign body giant cells have been produced as a direct response to these fractions, or indirectly through tissue irritation.

Reaction to Different Menstrua Used for Introducing Substances into the Peritoneal Cavity

We have never found multinucleated cells in the normal omentum (14). When distilled water (in R 709) or physiological salt solution (in R 730) is repeatedly injected into the peritoneal cavity of normal rabbits no evidence of irritation or cellular stimulation is seen on casual inspection. Microscopically a slight increase of phagocytic activity on the part of the clasmatocytes of the inter-milk spot areas may be noted, and among the monocytes normally present in milk spots there is an occasional epithelioid cell, mono- or binuclear. No giant cells of any type, however, are present. Consequently, these two

menstrua are the diluents of choice for the carrying of water-soluble substances into the tissues for an assay of their biological activity.

In the case of the fatty acid fractions and certain other of the lipoid fractions from the tubercle bacillus, it has, however, been necessary to use other vehicles. In the search for a bland non-irritating oil with which to dilute the more irritating corrosive fatty acids we have tested intraperitoneally olive and mineral oils among others. The reaction to repeated injections of olive oil has varied somewhat with the individual animal.

Olive oil has given a marked irritating effect, but the cellular response has ranged from a predominant reaction of clasmatocytes and neutrophilic leucocytes with numerous large foreign body giant cells (R 338, R 341) at the one extreme, to a mixed reaction in which monocytes, epithelioid cells, and rosette giant cells equalled or exceeded in number the cells comprising the less characteristic reaction (R 665, R 829; see protocols, page 57).

In Rabbit R 665 (see protocol, page 57) both types of giant cells were abundant; the foreign body type was very large with frequently more than 100 nuclei in a single cell; the epithelioid giant cells were relatively small with less than 20 peripherally placed nuclei. Careful comparative study failed to reveal any distinguishing differences in the size or character of the nuclei in the two types of giant cells which were often present in the same oil immersion field of the microscope. Monocytes with rosettes of normal size and character were numerous, and epithelioid cells were relatively fewer.

A comparative study of the character of the cytoplasmic bodies appearing in the two types of giant cells was provided through the mixed character of the cellular response to olive oil. Rabbits were injected at the same time with trypan blue by various routes and the cellular reaction to olive oil plus this vital dye was studied in unstained and supravitally stained preparations.

For example, Rabbit R 940 received nine daily intraperitoneal injections of 5 cc. each of olive oil, and during the same period by the same route seven injections, each of 4 cc. of a 1 per cent aqueous trypan blue solution, at 48 hour intervals. The animal was autopsied 12 days after the beginning of the experiment, at which time it was intensely blue, more especially in the tissues of the peritoneal cavity. The omentum showed considerable thickening at autopsy. On microscopic examination a great many giant cells were seen. Over half of the multinucleated cells contained unified central rosettes of fine trypan blue bodies with peripheral nuclei as illustrated in Fig. 1, while the remainder had scattered nuclei and larger segregation vacuoles similar to those seen in Fig. 2.

It is evident that the trypan blue in the epithelioid giant cell has been segregated into the "finely granular" apparatus, which was described originally by Langhans as a characteristic of the unstained epithelioid cells, and which the neutral red only makes more apparent as a typical cytoplasmic rosette. The trypan blue here makes prominent the apparatus of the cell in the same manner. When such cells as that shown in Fig. 1 were subjected to neutral red, introduced so that the process of staining could be watched with the oil immersion lens as it occurred, only the bodies already showing the trypan blue became purple and then violet as the neutral red superimposed itself upon the original dye. This phenomenon as just described for the rosette giant cells, applied in every particular to the reactions observed in the epithelioid cells of the same preparations, many of which contained the dye. In sharp contrast to the finely divided state of the trypan blue as found in the group of cells just mentioned, the numerous clasmatocytes or macrophages of the omentum were heavily laden with large, coarse, irregular aggregations of the dye, which had no typical pattern to their distribution in the cytoplasm. The large foreign body giant cells, likewise, showed scattered accumulations of blue dye, some of it finely divided but much of it in larger flocculations. There was evidence in some of the foreign body giant cells of an apparent fusion of mononuclear cells, identified in many instances as monocytes by individual rosettes still adjacent to their original nuclei. These observations lend support to the theory that foreign body giant cells are the product of the fusion of mononuclear cells many of which are of the monocyte type. The individual rosettes instead of fusing into one large central rosette to make an epithelioid giant cell lose their identity shortly after joining with others and the nuclei assume positions without an evident pattern within the larger cell. The bone marrow and lymph nodes of this animal showed only clasmatocytes (macrophages) taking up the trypan blue. The developing monocytes in the peripheral lymph nodes contained none of the trypan blue. This is in conformity with the observation that clasmatocytes and foreign body giant cells take up the dye much more readily than do the monocytes or epithelioid cells, which must be subjected to repeated direct contact with large quantities of the dye before showing the type of reaction just described, and shown in Fig. 1. The fixed sections

confirmed the findings disclosed by the supravital studies. Sections prepared and stained with Giemsa illustrated the unified central area of the epithelioid giant cells, whereas the cytoplasm of the foreign body giant cells gave evidence pointing toward their development as a result of the fusion of individual mononuclear cells, monocytes, and macrophages.

With mineral oil (Nujol), rabbits (R 660, R 661, R 662, R 663, R 1030, see protocols, pages 58, 59) varied in the amount of reaction to a given dosage as had those treated with olive oil (14); there was, however, never as much irritation with the former as with the latter.

Usually the peritoneal cavity showed little macroscopic evidence of reaction, the omentum having only a slight increase in number and size of milk spots. Both monocytes and clasmatocytes with occasional giant cells of both types represented the mild increase of cells found in the omentum. Most of the cells were filled with droplets of the injected oil, but there was little evidence of other embarrassment of the tissue in coping with the foreign material. In one animal (R 661) the predominant reaction was of clasmatocytes and fibroblasts with three giant cells of foreign body type to every epithelioid giant cell. No intermediate or equivocal types of giant cells were encountered. In going over one entire preparation from another rabbit (R 662), ten rosette cells with three or more nuclei were found to two foreign body types. In still another (R 663), six rosette giant cells containing from three to six nuclei were seen in covering some forty microscopic fields, and a like number of foreign body giant cells were encountered.

In general, the reaction to mineral oil was a mild, mixed one, clasmatocytes vying with monocytes for dominance, and these being accompanied by undifferentiated cells, and by giant cells of both types, containing oil droplets.

Giant Cell Reactions to Typical Foreign Bodies

To complete this survey of giant cell specificity, we included a study of the reactions, as analyzed by supravital stains, to three substances typically representative of the foreign body type, agar-agar, aleuronat, and lycopodium spores. The first material, while relatively resistant, can be eventually handled and probably completely absorbed and removed by the body cells. The second has in the past been used primarily to elicit a response of neutrophilic leucocytes through its property of sterile, chemical irritation. The third is a non-absorbable, foreign spore against which the tissues protect themselves without

elimination of the irritant. The relative sizes of the particles making up the two last mentioned substances may have influenced to some extent the differences observed in the respective reactions.

When sterile, warm, plain agar at pH 7.4 in a state that is just fluid is introduced in small amounts into the subcutaneous tissues it soon becomes well walled off with connective tissue. 7 days after the subcutaneous injection of agar into the abdominal wall at four points, and into the right groin, of Rabbit R 646, a biopsy (with ether) was performed and one of the abdominal areas excised for study. A scraping from the tissue about the agar showed that a great majority of the cells possessed granules or vacuoles of irregular size and shape, which were not distributed in any uniform pattern in the cytoplasm. Only a small percentage of the stained cells appeared to be monocytes though there were a few epithelioid types. Very few polymorphonuclear neutrophils and no lymphocytes were seen. Among the many foreign body giant cells were a considerable number of unstained cells of irregular shape, some branched and some with multiple nuclei. The character of these cells was not clear, and whether they were the precursors of the foreign body giant cells could not be determined from this tissue. The fixed sections of the tissue showed the breaking up of the agar by the invasion of strands of cells, most of which were in the form of large, foreign body giant cells, apparently attempting to surround and isolate smaller masses of agar.

7 days after the biopsy and 14 days after the agar injections the animal was killed and autopsied. The tissue from the inguinal region showed many mononuclear cells with hazy, amorphous granules or vacuoles such as had been seen in the biopsy material. There were many giant cells of the foreign body type with even sized nuclei scattered through the cytoplasm. Among the nuclei were many bodies both stained and unstained by the supravital dyes. A considerable number of clasmatocytes were present and many oil immersion fields contained from two to ten monocytes. The tissues from the subcutaneous areas on the abdominal wall were quite similar except for the presence of fewer monocytes.

Rabbit R 644 was injected intraperitoneally with 11 cc. of sterile, plain agar and in the abdominal musculature with 5 cc. 14 days later the animal was killed by the intravenous injection of air. A mass of sharply circumscribed agar was found in the abdominal musculature without evidence of reaction other than the connective tissue capsule immediately surrounding it. In the abdominal cavity were several small pieces of free agar, in addition to those embedded in the omentum. The latter organ was considerably thickened and moderately injected, but no general peritoneal irritation was present. The spleen was about twice normal size and on its surface were a few translucent nodules. No other gross abnormalities were detected.

Microscopic examination of the living cells around the agar in the abdominal wall showed the presence of many monocytes. Some typical epithelioid cells were encountered, a few with two, three, or more nuclei placed peripherally about

the central rosette. There were, however, many more of the foreign body giant cells found as a part of this reaction, the differential count revealing twenty-one of this type as contrasted with eight of the rosette or epithelioid type, the former being in each instance much larger than the latter when individual cells were compared. While the two types were usually not to be confused, as judged by the differential criteria previously given, occasional cells were found which did not belong clearly to either type.

The predominating cell in the scraping from the omentum was the monocyte. A few were seen with accentuated rosettes, the so-called epithelioid cells. Typical epithelioid giant cells were present, but they rarely contained more than four or five nuclei. On the other hand, there were a great many foreign body giant cells, most of which contained many nuclei, and much neutral red staining débris. Fig. 3 represents a cell taken from the omentum of this rabbit and illustrates the similarity which exists between this type of giant cell and those found secondary to other irritants, Figs. 2 and 8. The milk spots in a spread of the intact omentum were somewhat increased in size, but apparently not increased in number. Some of the milk spots were made up predominantly of clasmatocytes and others of monocytes. Both epithelioid giant cells and foreign body giant cells were present in some of the milk spots.

This experiment, as well as many others of similar nature, has convinced us that when bland substances are introduced, giant cells of either of the types under consideration may be found without necrosis. Under pathological conditions it is probable that foreign body giant cells are only formed secondarily to areas of necrosis or caseation.

A differential count of the peritoneal fluid from the animal under discussion showed 74 per cent monocytes, 15 per cent clasmatocytes, 3.5 per cent lymphocytes, 3.5 per cent neutrophils, 3 per cent serosal cells, and 1 per cent unclassified cells with a total count of 5,600.

A study of the fixed tissue in section showed again the agar infiltrated with cells and in the process of being broken up into fragments of various size and shape. Many of the smaller fragments were completely surrounded by giant cells, which were for the most part very large, with nuclei scattered without pattern through the cytoplasm. The areas of the omentum which were free of agar were entirely normal and uninvolved in the local reaction. The other abdominal organs showed no significant changes from the normal.

The recognition in these preliminary studies of a phase of monocyte stimulation in the general tissue reaction to agar suggested to one of us (12) the possibility of producing, if sufficient foci could be established, a monocytosis in the peripheral blood similar to that observed in tuberculosis.

Rabbits were injected with as much as 40 cc. of agar in multiple areas subcutaneously and intraperitoneally. The anticipated monocytosis developed, but, in addition, the reaction in the tissues was further reflected in the blood through the appearance of giant cells in the peripheral circulation. This is, so far as we know, the first time that such a phenomenon has been produced experimentally. Forkner (12) has made a detailed study of the blood and tissues under these conditions; the opportunity thus afforded for observation of selected giant cells in the blood stream provided convincing evidence that the foreign body type of giant cell arises from the fusion of cells.

Aleuronat, another substance which induces a mixed reaction in the tissues, causes the production of about equal numbers of foreign body and epithelioid giant cells. Indeed, it has proved one of the best substances to employ for comparative studies of the two types of giant cells, both types being frequently found in the same milk spot of the omentum and in the same oil immersion field.

Figs. 11 and 12 are photographs of the two typical kinds of giant cells as found in adjacent microscopic fields from the omentum of Rabbit R 813. Seven intraperitoneal injections of 0.5 gm. each of aleuronat in 10 cc. distilled water were given and the reaction studied 1 day after the last injection. There was no free fluid in the peritoneal cavity. Fibrinous adhesions bound certain of the intestinal loops. The omentum was thickened, hyperemic, and showed an increased number of milk spots. Microscopically the serosal cells showed marked irritation and there were many polymorphonuclear leucocytes infiltrated diffusely and in abscesses of various sizes. Monocytes, epithelioid cells, and epithelioid giant cells abounded, though without any tendency for the formation of aggregations of cells similar to those characteristic of tuberculosis. Fig. 11 illustrates several epithelioid giant cells in this reaction. All show the central rosettes, dark in the photograph, and, in one instance, an entirely clear centrosphere is conspicuous in the center of the mass of fine neutral red bodies. This area of rarefication in the vacuoles at the center of the mass is usually visible at some level when an entire cell of this type is subjected to a study at different points of focus with the oil immersion lens. Associated with the localized abscesses were many large, highly phagocytic clasmatocytes and foreign body giant cells. In many places these very large giant cells with scattered nuclei were on the edge of a definite area of necrosis; in other places, however, they were remote from any visible necrosis. Fig. 12 shows the scattered nuclei and the relatively diffuse peripheral localization of the neutral red staining material, which characterize the foreign body giant cell. Many plasma cells were present, which frequently occur in association with healing in areas of chronic irritation.

In every animal which received aleuronat, two very distinct types of giant cells could be identified and distinguished the one from the other in the fixed tissues as well as in the supravital preparations.

It is, of course, well known that *lycopodium spores* produce a reaction in the tissues, the major cellular constituent of which is the large, phagocytic, multinuclear giant cell.

Rabbit R 732 was injected intraperitoneally and subcutaneously 2 weeks prior to autopsy with lycopodium spores suspended in distilled water. When examined, the peritoneal fluid, which was increased in amount, showed a differential count of 86 per cent monocytes, 12 per cent neutrophilic leucocytes, and 1 per cent each of lymphocytes and serosal cells. The omentum was the principal organ dealing with the injected material as was shown by the presence of many brownish areas where the spores were aggregated into clumps. Microscopically the spores were seen to be within giant cells of varying size, either singly or in small groups, some individual cells covering from two to three oil immersion fields. The spores occupied a central or excentric position in these cells with nuclei scattered about in the cytoplasm. Very few of the giant cells contained any cytoplasmic bodies which reacted with neutral red or Janus green. Thus, the cell of Fig. 8, Plate 9, was drawn from a preparation in which the two supravital dyes had stained other cells in the same field without showing any specificity for the finely granular cytoplasmic structures in the giant cell. This lack of staining has been a characteristic common to a certain proportion of foreign body giant cells whenever found. Many of these multinucleated cells seemed to have been formed by fusion of several cells combining about a body too large for a single mononuclear cell to engulf. There was otherwise a surprising absence of cellular reaction. Eosinophils and plasma cells infiltrated the reaction area to a limited extent. While there were both clasmatocytes and monocytes in the tissues, few epithelioid cells and neutrophilic leucocytes appeared as part of this 14 day old reaction. An occasional small rosette cell with two to five nuclei was seen, but in no instance were these cells phagocytic for the lycopodium spores.

If these giant cells were formed from monocytes, the cytoplasmic pattern either had been lost or was not detectable in our studies. It is entirely possible, and we believe probable, that these giant cells were formed from primitive connective tissue mesenchymal cells, undifferentiated at least to the degree that they showed no characteristic cytoplasmic structure reacting with neutral red.

The tissues from the subcutaneous areas where spores had been injected showed a reaction in all respects similar to that seen in the omentum, except that there were fewer monocytes. This discrepancy

between areolar connective tissue in general and the omentum with respect to the quantitative response of monocytes under various conditions, indicates that the latter organ is a somewhat readier source of the rosette cells. This may be because of the milk spots of the omentum, many of which normally contain monocytes.

DISCUSSION

It is clear that the giant cells which form in the tissues in response to irritants may be divided in general into two types, one with scattered nuclei and no characteristic cytoplasmic pattern, and the other with nuclei arranged about a central cytoplasmic rosette. The fact that both types have been found repeatedly in close association within the same lesion has tended, however, to minimize the significance of such a classification as long as it has been based on nuclear distribution alone. Nevertheless, the frequency with which giant cells with nuclei arranged around a clear center have been found in the lesions of tuberculosis since Langhans' first description, has caused their predominance to be regarded as characteristic of this disease, this notwithstanding the fact that such giant cells have also been found in more or less abundance in many other pathological conditions.

It is now essential to consider all the histological criteria available for analyzing these giant cells of the connective tissues. The first point for consideration is that of the relative size of the individual cells. Never have we seen a really large giant cell with a single central rosette of neutral red bodies, that is to say, one which contained more than twenty to twenty-five nuclei; the great majority contain from six to ten nuclei with a corresponding limitation in the size of the cell. While an individual foreign body giant cell with scattered nuclei and no rosette may be equally small, the majority of these cells found in any reaction are relatively large, some cells having been seen which covered three or more oil immersion fields of the microscope with from 100 to 300 nuclei. Thus, if a given giant cell seen in fixed tissue is very large, it probably would not have shown a single central rosette in the supravital preparation and thus should not be identified with the epithelioid type as defined in Chapter I on pages 8 and 9. Apart from arrangement, no structural differences in nuclei, such as in chromatin or in nucleoli, have been detected. On the other hand,

the finer histology of the cytoplasm does yield additional evidence upon which to base differences in type.

The nature and the function of the bodies which stain with neutral red and which make up the rosette seen in the living monocyte are still matters for discussion and speculation. The much disputed relationship between the rosette cells and other closely analogous cells in the connective tissues can only be ultimately understood by analyzing the function of these bodies. These visible structures within the cytoplasm of the monocyte have neither the definiteness nor the solidity of the granules found in the polymorphonuclear leucocytes. Moreover, they may be seen to change in individual size while under observation, much as do the vacuoles of digestion in the amoeba or the phagocytic vacuoles of the clasmatocyte. They are thus of the nature of vacuoles rather than granules, but, unlike the vacuoles of the cells mentioned, which develop only for the segregation of the phagocytized objects, they seem to be an inherent part of the cell structure, appearing during the maturation phase from monoblast to monocyte. The mature monocyte always shows these vacuoles as a visible mechanism, apparently indigenous to the cell and prepared for the function of dealing with certain types of débris. Moreover, these vacuoles, whether in the normal or the stimulated monocyte, or in the epithelioid cell, appear to remain at relatively the same pH, as far as that can be judged by the reaction to neutral red. It may be that these vacuoles always contain as a part of their normal condition certain metabolic products with which the cell is dealing physiologically but which we have at present no means of detecting.

Thus far the study of phagocytic activity has been dependent on the recognition of substances, such as whole cells, or artificially injected particles, which by a period of maintained identity within the cell, can be followed with certainty. When such obvious débris is found phagocytized within rosette cells, it is contained within the vacuoles of the cytoplasm and usually shows the same reaction to neutral red as normally characterizes the entire rosette. In the epithelioid cell these vacuoles are greatly increased in number and frequently decreased in size, giving a much enlarged rosette. The same massing of vacuoles characterizes the epithelioid giant cell, and the very fine, dust-like mitochondria, which characterize the rosette cell in contrast

to the larger bodies staining with Janus green in the lymphocytes, are conspicuous in the giant cell of this type. Thus it appears that epithelioid cells and the corresponding giant cell lack the power, so conspicuous in the clasmatocyte, of segregating into large masses particulate matter which has been phagocytized. Moreover, when, under the overwhelming doses of experimental conditions, the cells have been forced to take in large masses of the phosphatide, this is quickly dispersed into fine particles within the cell.

The studies with the tuberculo-phosphatide indicate a formation of epithelioid cells as result of the phagocytosis of this material and show that the corresponding giant cell is merely a multinucleated epithelioid cell. Consequently, any condition which tends toward the production of the epithelioid cell, likewise tends to give rise to these giant cells. It thus appears that the epithelioid giant cells, identical with the so-called Langhans type, make a distinct group. In this group each giant cell is a direct derivative of one epithelioid cell, usually through amitotic nuclear division. As far as our data go, the tuberculo-phosphatide and certain fatty acids from the tubercle bacillus, together with lecithin in a minor degree, have given epithelioid cells. Many other lipoids, on the other hand, have been negative in this respect.

The formation of giant cells of the foreign body type is much more complex. They have followed the introduction of a wider range of substances, and none of the reactions in which they have appeared have been entirely free from the indirect effects of tissue irritation. Evidence has been presented, especially through the recent work of Forkner (12) to indicate that this type of giant cell comes not from the transformation of a single cell, but from the fusion of many. Such a method of formation will naturally result in giant cells showing great variation in size.

Differences in the structure of the giant cell will occur if different types of cells are involved. In our experiments it has been predominantly monocytes which have fused to form foreign body giant cells. The occasional fusion of one or more epithelioid cells with monocytes, or of monocytes with clasmatocytes results in more complex giant cells. There is evidence that primitive connective tissue cells may also fuse to give rise to giant cells. The definitive cells of the blood and

connective tissues arise throughout life from primitive anlagen through a period of maturation, and when the stimulus or the demand is sufficiently great, the immature forms function in so far as they are able in the attempt to meet the emergency. When a reaction in the connective tissues reflects an increased production and mobilization of cells, it is possible that a direct formation of giant cells from the newly stimulated undifferentiated connective tissue cells may occur. Such an explanation as this seems probable in the case of the subcutaneous injections of agar, in which non-rosette giant cells appeared in abundance associated with mononuclear cells less differentiated than the monocyte, and in the reaction about the lycopodium spores, which were found within cells taking no neutral red dye and with few typical monocytes in the tissues.

While experiments with bland foreign bodies such as agar have shown that foreign body giant cells may be artificially produced without apparent necrosis, the destruction of tissue in pathological processes is probably the usual stimulus to them. It is possible that while the phosphatide and fatty acids of the tubercle bacillus stimulate only the epithelioid types, the waxes may be liberated from the bacilli in a state in which they might lead to the formation of the foreign body types.

CONCLUSIONS

1. There are two types of giant cells discriminated by their method of formation; one is derived from a single monocyte or epithelioid cell and the other by the fusion of cells.

2. The epithelioid giant cell is formed by amitotic nuclear division; it tends to be small, simple, and relatively uniform in structure; its essential structure is a rosette of fine vacuoles which in the living state are stainable with neutral red.

3. The foreign body types show extreme variation in size and structure, according to the nature and the number of the cells out of which they are made. They lack any constant cytoplasmic pattern.

4. Both of these types of giant cells are probably formed in response to the need for phagocytosis of foreign material.

5. In tuberculosis it is probably only certain specific portions of the

lipoids that induce the production of mono- and multinucleated epithelioid cells.

6. The stimuli for the formation of giant cells of the foreign body type are much more varied than those which produce epithelioid giant cells.

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EXPLANATION OF PLATES

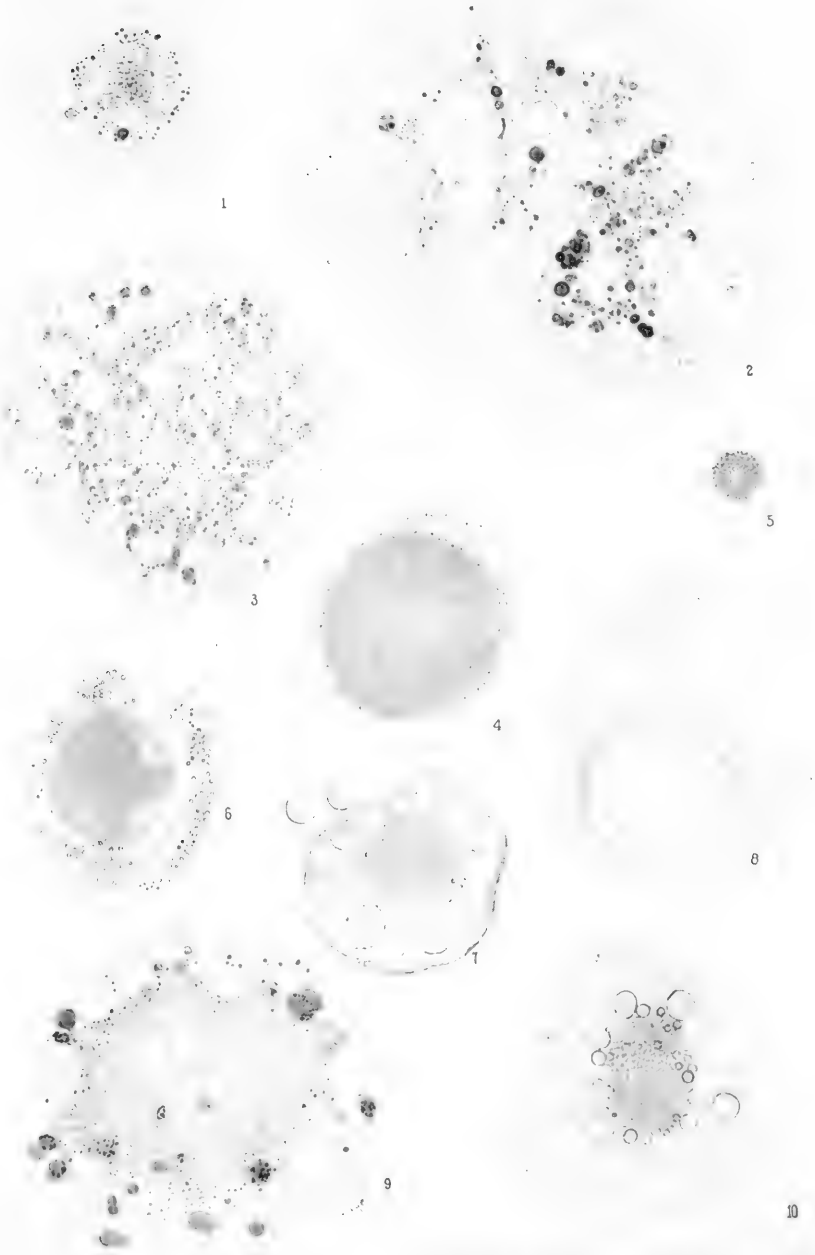
PLATE 9

FIG. 1. An epithelioid giant cell from the omentum, vitally stained with trypan blue, from Rabbit R 940, which had nine intraperitoneal injections of 5 cc. olive oil and seven injections of 4 cc. of 1 per cent suspension of trypan blue. Size 52.3 x 50.8 microns.

FIG. 2. A foreign body giant cell from the same animal as Fig. 1. Notice the lack of uniformity in size of the vacuoles and the absence of pattern in the arrangement of the nuclei and cytoplasmic structure. Size 106.6 x 90.2 microns.

FIG. 3. A foreign body giant cell from the omentum of Rabbit R 644. This animal had received 11 cc. of agar intraperitoneally. Stained supravitally with neutral red. Size 75 x 70 microns.

FIG. 4. Epithelioid giant cell from the omentum of Rabbit R 684. This animal had received twelve intraperitoneal doses of 80 mg. each of the phosphatide A-3 from avian tubercle bacilli. The rosette is unusually large and the nuclei are



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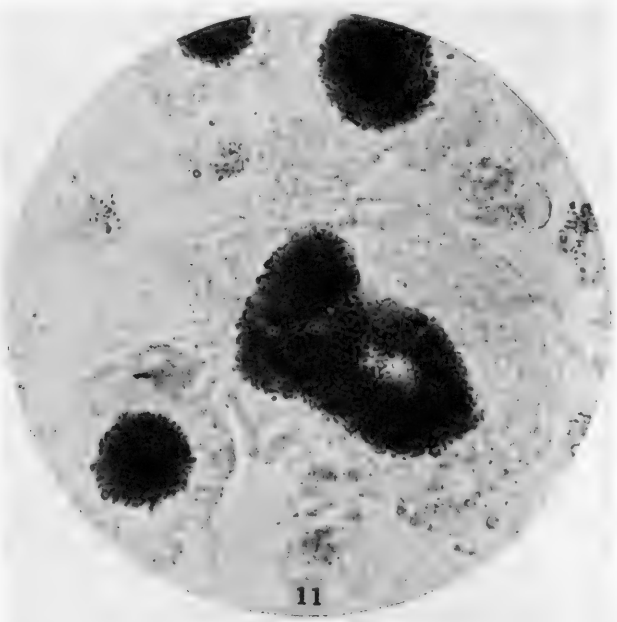

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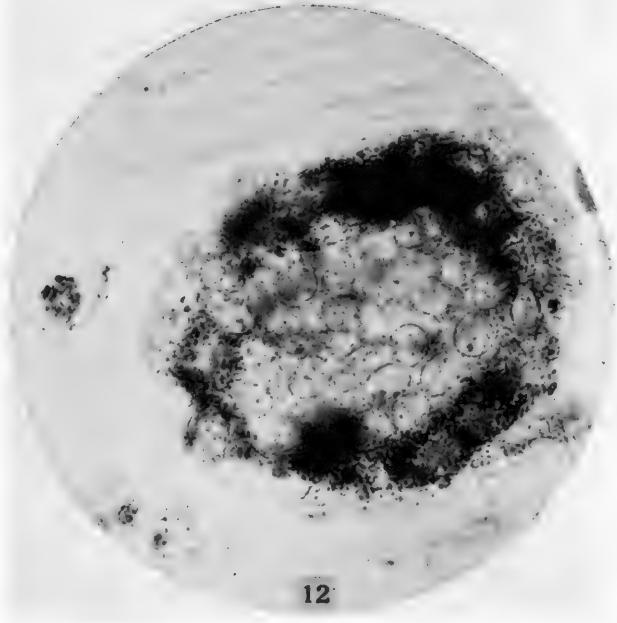

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(Doan, Sabin, and Forkner: Derivation of giant cells)

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crowded far out to the periphery of the cell. Stained with neutral red. Size 47.8 microns in diameter.

FIG. 5. Mononuclear epithelioid cell from same specimen as Fig. 4. Size 8 x 12 microns.

FIG. 6. Epithelioid giant cell from Rabbit R 918 which had received twelve intraperitoneal doses of 20 mg. of fatty acid IV from tubercle bacilli H-37. Stained with neutral red. Size 72.7 x 52.5 microns. This cell shows many refractile bodies in the periphery. The nuclei are not as sharply peripheral as is usually the case.

FIG. 7. A typical epithelioid giant cell from the tibial bone marrow of Rabbit R 1124 dying of bovine tuberculosis. Note the evidences of amitotic nuclear division and the dust-like character of the bodies staining with neutral red, making up the central rosette. The two cells just above are small lymphocytes for comparison in size. Size 42 x 42 microns.

FIG. 8. Foreign body giant cell completely engulfing a lycopodium spore. This cell took no dye, though other cells in the same field were stained. It was one of the smallest of the giant cells seen in this reaction. The animal, R 732, had received lycopodium spores subcutaneously and intraperitoneally. Size 73.8 x 31.4 microns.

FIG. 9. Epithelioid giant cell from lung of Rabbit R 844 which had received three intravenous injections of 10 mg. each of phosphatide from H-37. One nucleus is centrally placed and can be seen to overlie the cytoplasmic structures, thus obscuring, at the focus of the drawing, those immediately beneath. Note carbon particles within some of the neutral red vacuoles. Stained with neutral red. Size 66 x 59.7 microns.

FIG. 10. Epithelioid giant cell from omentum of Rabbit R 921, which had received twelve intraperitoneal doses of 20 mg. of fatty acid V from tubercle bacilli H-37. The nuclei are in parallel layers closely encapsulating the rosette. Stained supravitally with neutral red, but with some of the vacuoles showing no reaction to the dye. Note the larger size of the individual vacuoles at the periphery of the rosette. Size 62.8 microns in diameter.

PLATE 10

FIG. 11. Several epithelioid giant cells from Rabbit R 813 which had received intraperitoneal injections of aleuronat. Photograph is of cells *in situ* within the omentum, stained supravitally with neutral red. The darker bodies represent the cytoplasmic reaction to the dye. Note the contrast between the arrangement of the neutral red vacuoles in a rosette with peripherally placed nuclei and the lack of any pattern in vacuoles or nuclei in the foreign body giant cell of Fig. 12. Magnification × 830.

FIG. 12. Foreign body giant cell from same omentum as Fig. 11. Note the scattered nuclei and lack of pattern in the cytoplasmic structures. The increased intensity of the neutral red reaction is also evident. Magnification × 830.

CHAPTER IV

THE RELATION OF THE TUBERCLE AND THE MONOCYTE-LYMPHOCYTE RATIO TO RESISTANCE AND SUSCEPTIBILITY IN TUBERCULOSIS

By CHARLES A. DOAN, M.D., and FLORENCE R. SABIN, M.D.

INTRODUCTION

Interest in the pathology of tuberculosis has centered upon the tubercle, of which the epithelioid cell has been recognized as an essential and characteristic unit. In the discussions on immunity in this disease, tubercles have also been deemed important, for it has been usual to consider the tendency toward their formation as a manifestation of a cellular resistance on the part of the body. The studies reported here represent an attempt to test this view by experiment.

Baumgarten (1) first established the fact that the tubercle is an intervascular structure and showed that the epithelioid cell is due to a proliferation of fixed tissue cells. Recently, Sabin, Doan, and Cunningham (2) found that the epithelioid cell was related to the monocyte, which is a cell both of the connective tissues and of the circulating blood. It was then shown by Cunningham, Sabin, Sugiyama, and Kindwall (3) that in bovine tuberculosis, experimentally induced in rabbits, the monocyte-lymphocyte ratio in the peripheral blood might be taken as an index of the progress and extent of the disease. They found that circulating monocytes increase in number with dissemination, and decrease with regression of lesions, and that a lessened number of lymphocytes accompanies susceptibility and that they increase with resistance. Camp, Luton, Tompkins, and Cunningham (4) confirmed these findings in guinea pigs infected with a human strain of tubercle bacilli. The experimental work has also demonstrated that qualitative changes in the monocytic strain of cells are significant, the presence of altered monocytes and of typical epithelioid cells in the blood stream, even in the absence of striking numerical changes, being indicative of progressive tuberculosis.

Clinical evidence has now been added which confirms the importance of an increased or a reversed monocyte-lymphocyte ratio and of the presence of epithelioid cells in the blood as a means of following the course of the disease (5–13).

Morriss and Tan (5) reported the first series of human cases in which the period of extending lesions was correlated with a high monocyte-lymphocyte ratio. A second series of cases were analyzed by Cunningham and Tompkins (6) who showed the correlation between a high index and extending lesions and between a low index and resistance. Rogers (7) found that modified monocytes and epithelioid cells are almost constantly present in the blood of children with tuberculous lesions, that a high monocyte-lymphocyte ratio is correlated with extending lesions, and that epithelioid cells may be present in the blood of children with incipient tuberculosis before the intradermal tuberculin test becomes positive. Blackfan and Diamond (8) consider that in children the presence of epithelioid cells in the blood and a reversed monocyte-lymphocyte index may be more significant than the tuberculin reaction. The same point has been brought out by Pittaluga and Garcia (12), who followed the clinical course of babies after the oral administration of *Bacillus Calmette-Guerin*, and found a rise in monocytes by the 6th day, which was either accompanied, or followed by an increase of lymphocytes and a decrease in neutrophils. These phenomena they interpreted as an indication of a mild infection with subsequent development of resistance. Further clinical studies have been made by Wilkins (9) and by Flinn (10, 11).

Doan and Sabin (14) also demonstrated that indications of the extent and progress of experimental tuberculosis may be obtained not only from changes in the mononuclear cells, monocytes, and lymphocytes; but also from variations in the elements derived from the bone marrow. They found that with massive intravenous infection, a temporary involvement of the bone marrow induced an anemia and a fall in granulocytes and platelets. This condition was followed by a hyperplasia of the bone marrow whenever the animal survived the acute phase of the infection. Medlar (13) has pointed out from a study of clinical cases the importance of analyzing the entire blood picture; for beside the increase in the ratio of monocytes to lymphocytes when tubercles are extending, the onset of caseation in the tissues is reflected in the blood by a rise of neutrophilic leucocytes.

The value of the studies of the blood as indicating the course of the infection plainly resides in the fact that the blood cells reflect changes in the lesions in the tissues; but the significance of the different elements in the lesions, with reference to variations in susceptibility to the disease, is not yet clear. For example, the association of increased resistance and a high lymphocyte count was noted by Webb *et al.* (15) and was experimentally demonstrated by Murphy (16) before the relationship to the fall in monocytes was known; but how the increase in lymphocytes acts in increasing resistance is as little understood as when the observations were first made.

An analogy has been drawn between the increase in monocytes in tuberculosis and that of neutrophils in pyogenic infections, whence the inference that the increase in monocytes is a means of defence against the invasion of tubercle bacilli. There are, however, certain facts known of tuberculosis which make this inference unjustifiable. Evidence from the study of living monocytes containing tubercle

bacilli (3), and from tissue cultures grown with them (17) indicates that tubercle bacilli survive and may even multiply within the monocyte and its derivative, the mono- and multinucleated epithelioid cell. It is known that phagocytized material of any sort with which cells can deal effectively is characteristically to be found within vacuoles which stain supravitally with neutral red. This is true both of various kinds of bacilli which are taken in by leucocytes and of leucocytes themselves when engulfed by clasmatocytes. The vacuoles have been interpreted as a manifestation of the mechanism of the cell for the digestion of such material. Tubercle bacilli seen within living mono- and multinucleated epithelioid cells are not surrounded by vacuoles. This fact and the presence of the large numbers of bacilli found in epithelioid cells suggest a host-parasite relation between monocytes and tubercle bacilli. Acceptance of such a view would give quite a different significance to the phagocytosis of tubercle bacilli by monocytes from that of the phagocytosis of other bacilli by cells which kill and digest them. Evidence in sections as to whether bacilli were living or disintegrating within the epithelioid cells is, of course, inconclusive, and is complicated by the fact that there are two kinds of granules of tubercle bacilli, the acid-fast débris of disintegrating organisms and the so-called "Much" granules or minute living stages in the life cycle of the bacillus demonstrated by Kahn (18). The dissociation of tubercle bacilli by Petroff *et al.* (19, 20) into rough and smooth colonies of different virulence, and the studies of Kahn (18) on the life cycle of tubercle bacilli both undissociated and dissociated, show that the relationship between the bacillus and the monocyte may be more complex than a mere survival or even multiplication of the organism within the monocyte. It is not impossible that a change in the type of the bacillus, involving a change in virulence, may take place while the organism is within the epithelioid cell.

During the past 5 years we have attempted to accumulate experimental data concerning the cellular factors of host resistance in tuberculosis. While so doing we have followed the period of survival in animals infected with a uniform dose of an acid-fast organism of a standard strain under experimental conditions which have induced a change in the numbers of monocytes in the blood and tissues. The criteria of extent of disease and survival have been the same as those employed by Petroff, Branch, and Jennings (21) in their immunological studies with heat-killed bacilli. As resistance in tuberculosis is only relative, these authors strongly emphasize the necessity of much smaller doses of infecting organisms than we have used in the analysis of host resistance. Our own studies have fully convinced us of the validity of this point. Nevertheless we have used large doses, since any variations in resistance under the adverse condition of an overwhelming dose of organisms must be considered significant.

The Relation of the Pre-Infection Monocyte-Lymphocyte Ratio to Survival in Experimental Tuberculosis

It has long been known that there are marked differences in the resistance of animals of the same species to an identical dose of tubercle bacilli. Having found that the degree of resistance to the course

TABLE IX

The M/L Index and the Period of Survival of Rabbits after 1–2 Mg. Bovine B-1 Tb.

No. of rabbits	Treatment or condition	Average pre-infection M/L index	Average period of survival after tb.
			days
50	All normal stock	0.280	47
	42—survived less than 70 days	0.298	28
	13—M/L index above 0.300	0.580	33
	29—M/L index 0.300 and below	0.171	27
	8—survived more than 70 days	0.186	145+
13	All rabbits receiving any dosage whatever of antimonocytic sera (see Table XI)	0.190	97+
6	Rabbits treated with antimonocytic sera (excluding those with high monocytes in acute phase of disease at time of serum injections and those with toxic dosage) (R 14, R 15, R 69, R 72, R 46, R 52)	0.161	154+
2	Normal rabbits given normal rooster's serum in dosages comparable to those received by the last six rabbits above	0.262	59
7	Received intraperitoneally phosphatide A-3 (240–1,040 mg.) previous to inoculation with tb. (see Table XII)	0.652	31
7	Normal, receiving same amount of same culture suspension as above	0.189	61+

The abbreviation tb. means tubercle bacilli; tbc., tuberculosis.

of an infection could be measured by the ratio of monocytes to lymphocytes in the blood, it seemed worth while to see if differences in the monocyte-lymphocyte ratio of animals before infection might be correlated with longevity after infection.

In the first section of Table IX are shown the pre-infection mono-

cyte-lymphocyte ratios of fifty normal rabbits and their period of survival after the intravenous injection of 1 to 2 mg. of bovine tubercle

TABLE X

Data for the 50 Rabbits in the First Section of Table IX

Thirteen rabbits, high index, low resistance		
Animal No.	Pre-infection M/L index	Survival
		days
R 605	1.200	22
R 103	1.000	69
R 90	0.900	37
R 609	0.580	25
R 601	0.570	22
R 101	0.555	49
R 196	0.487	36
R 186	0.458	32
R 537	0.430	30
R 476	0.350	26
R 600	0.350	25
R 40	0.333	27
R 232	0.333	35
Average....	0.580	33

Eight rabbits, low index, high resistance		
R 100	0.318	206
R 475	0.263	78+
R 102	0.230	195
R 412	0.186	119+
R 185	0.185	142+
R 11	0.140	110+
R 38	0.096	236
R 477	0.071	78+
Average....	0.186	145+

Twenty-nine rabbits, low index, low resistance		
Animal No.	Pre-infection M/L index	Survival
		days
R 48	0.300	34
R 82	0.270	60
R 58	0.261	31
R 608	0.260	18
R 42	0.258	20
R 50	0.241	21
R 83	0.237	16
R 411	0.215	30
R 13	0.210	19
R 32	0.200	21
R 21	0.190	30
R 30	0.170	42
R 18	0.170	20
R 41	0.166	20
R 71	0.166	31
R 76	0.163	32
R 535	0.162	26
R 603	0.160	21
R 20	0.160	25
R 37	0.155	15
R 602	0.150	22
R 16	0.150	35
R 70	0.140	25
R 515	0.130	31
R 61	0.102	25
R 34	0.06	21
R 35	0.06	20
R 75	0.04	38
R 60	0.03	29
Average....	0.171	27

bacilli, Strain B-1. Of these animals, forty-two or 84 per cent died or were killed *in extremis* before 70 days had elapsed. The data for the fifty rabbits are given in Table X. The animals are arranged

in three groups, the order in each of which is that of decreasing M/L indices. In the first group are the rabbits, thirteen in all, with the highest indices, ranging from 1.200 to 0.333, average 0.580, and it will be noted that they were all in the group with low resistance, as shown by an average survival period of 33 days. All the rest of the animals showed indices with a range of 0.318 down to 0.030, of which eight showed high resistance, with an average period of survival of 145+ days, and twenty-nine showed low resistance, survival 27 days. The data then indicate that the expectancy of life after infection was consistently short in the animals with an index above 0.318, while no prediction could be made concerning those with an index below 0.318. It also will be noted that the length of survival was not proportional to the index; for example, Rabbit R 232, with an index of 0.333, survived 35 days, while R 100, with an index of 0.318, survived 206 days. In obtaining the pre-infection index for this group of animals, a series of seven counts before infection was the rule, but there were some in the series in which only one count was taken. To make a more adequate study of this point, we have infected a series of rabbits which have been selected with reference to the M/L index. In obtaining this series we found that the animals fell into four groups: one in which the indices were consistently high as determined by repeated counts; one consistently low; one average; and a group in which the counts varied widely from day to day. The results of this study will be reported later.

The Course of Tuberculous Infection in Rabbits Injected with an Antiserum for Rabbit Monocytes

The experiments which follow were undertaken on the assumption that the relation of monocytes to lymphocytes in the peripheral blood is a reflection of the relative number and availability of these two types of cells in the tissues and organs of the body. The attempt was made to modify this ratio, first through the blood directly and secondly through the tissues. Under these conditions we have studied the subsequent course of a primary tuberculous infection. The experiments under discussion concern a modification of the ratio of monocytes to lymphocytes in the blood.

The attempt was made to procure a specific antiserum for rabbit

monocytes, similar to those already known for blood platelets (22), lymphocytes (23), and red blood cells (24). It was hoped that by the injection of this serum the number of monocytes in experimental tuberculosis could be lowered at will.

As a source of the serum, Black Jersey Giant roosters were used, and the lungs of tuberculous rabbits were utilized as antigen.

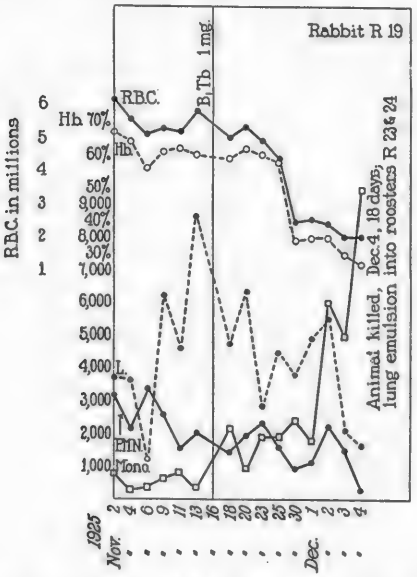

CHART 5. Blood counts of Rabbit R 19, killed 18 days after infection, showing development of a high M/L index which was correlated with extreme tuberculous consolidation of the lungs. Animals yielding this type of reaction to infection were selected for the preparation of lung emulsions for antigenic inoculation into roosters.

A certain percentage of rabbits inoculated with 1 to 2 mg. of B-1 strain tubercle bacilli develop within the first 3 weeks after infection a massive bilateral pulmonary consolidation. Macroscopically the lungs are firm, grayish yellow, and non-crepitant; microscopically only a few alveoli remain patent; diffusely scattered epithelioid cells and some leucocytes obliterate most of the normal structure of the organ. Here and there is a beginning microscopic area of necrosis, but no extensive caseation has yet developed. The condition in the lung corresponds to the human gelatinous tuberculous bronchopneumonia. This stage of the disease

is marked by a striking rise in the monocytes of the peripheral blood (see Chart 5). We exsanguinated rabbits showing such a typical rise of monocytes in the peripheral blood, and made sure of the tuberculous nature of the pulmonary consolidation by supravital studies. The lungs, removed under aseptic precautions, were carefully freed of extraneous tissue, cut into small pieces, and ground in a mortar with sterile sand. Enough physiological salt solution was added to make 40 cc. of the final emulsion, and 20 cc., representing one-half the total consolidated lung tissue from one rabbit, were injected intraperitoneally into each of two Black Jersey Giant roosters. Four roosters (R 23, R 24, R 27, and R 28) were given six injections each at approximately weekly intervals. Serum was taken from two of the roosters, R 23 and R 24, 17 days after the last injection, and all four were exsanguinated in 29, 38, 17, and 25 days respectively. Two roosters (R 21 and R 25) were the source of the normal serum used as control.

The antigenic material injected into the roosters was, of course, of heterogenous nature, but the epithelioid cells greatly predominated and the results showed a greater effect on the monocytic strain of cells than on the other types of white blood cells. The roosters had complete native immunity to the bovine tubercle bacilli introduced with the lung emulsions, as was proven by the absence of tuberculous lesions at autopsy.

In a recent study, Sabin, Miller, Doan, and Wiseman (25) have shown that injections of tuberculo-proteins and polysaccharides give rise to constant changes in the white blood cells. The same reactions follow the injection of a wide range of chemical substances including bacterial proteins (26–29) and various salts (30). As finally analyzed, the phenomenon consists of an immediate fall in all three of the major groups of the white blood cells, neutrophils, lymphocytes, and monocytes, with a subsequent rise of each group starting at different times. It was shown by Beard and Beard (31) that the fall in the neutrophilic leucocytes after the injection of salt solution was very transient, the rise beginning within a half-hour. After injections of the tuberculo-protein and polysaccharide (25) the lymphocytes suffered the most prolonged fall; they remained at a low level for 7 hours, the period of the observations, and returned to the original level only during the following night.

In this series of experiments, fourteen rabbits, listed in Table XI, received 54 injections of treated rooster serum, in doses as shown in the table. Blood counts were made during the day after 39 of these injections, and the effects on the blood cells are given in the following analysis. Six injections of normal serum were given as controls;

five of these were in the tuberculous rabbits (R 40, R 43, R 46, and R 87) and one in a normal rabbit (R 46 before infection). There

TABLE XI

The Tuberculosis of Rabbits Receiving Specific Antisera for Rabbit Monocytes

Number	Antiserum given before 1 mg. B-1 tb. intravenously	Antiserum given after infection	M/L index before infection		M/L index of last count before death	Died	Killed	Tuberculosis post mortem
			Before serum	After serum		days	days	
R 14	20 cc. in 7 doses	—	0.250	0.083	1.143	380		+++ chronic with terminal miliary
R 15	" "	—	0.109	0.073	3.750		96	+++ chronic
R 45	1 cc. in 1 dose	—	0.322	0.182	0.813	165		+++ "
R 46	0.9 cc. in 1 dose	11 cc. in 5 doses	0.809	0.500	2.316	150		+++ "
R 52	1 cc. in 1 dose	5 cc. in 2 doses	0.150	0.135	3.777	168		+++ "
R 69	20 cc. in 7 doses	—	0.194	0.088	2.700		64	++ "
R 72	" "	—	0.470	0.071	1.800	68		+++ "
R 68	2.5 cc. in 1 dose	—	0.096	0.036	1.190	59		+++ chronic
R 40	—	0.9 cc. in 1 dose	0.333	—	0.777	27		+++ acute
R 43	—	4 cc. in 4 doses	0.143	—	0.695	22		++
R 59	—	6 cc. in 4 doses	0.087	—	0.882	22		+++ and pn.
R 77	20 cc. in 2 doses	—	0.577	0.357	2.215	21		+++ acute
R 78	" "	—	0.400	0.400	1.571	19		+++ "
R 79	" "	—	0.250	—	Died immediately after second injection			—

were two injections of treated serum inactivated by heat and one of inactivated serum plus normal serum. The results of the injection of inactivated serum will be illustrated under the special cases.

In general, after the injection of normal rooster serum into the rabbits, the blood cells reacted as after the injection of other types of protein with certain minor exceptions. The neutrophilic leucocytes fell and then rose above their original level in four out of six cases.

The first exception was after an injection in Rabbit R 46, in which there had been an injection of the treated serum the day before and the neutrophilic leucocytes were still at a high level, 18,022 per c.mm. before the injection of the normal serum. They fell to 10,894, and then rose only to 14,284 cells during the day. The second exception was in Rabbit R 40, in which the neutrophilic leucocytes fell but did not rise to the original level. The animal died in 2 days of tuberculosis, and its condition probably accounted for the lack of response from the bone marrow.

After normal rooster's serum, both monocytes and lymphocytes fell, the former 53.37 per cent and the latter 35.02 per cent. The percentage of fall or rise has been computed by considering the original level of the cell under consideration as unity. The fall in monocytes in the normal rabbit was 35.90 per cent and the range in the tuberculous rabbits was from 13.78 to 86.50 per cent.

The most striking reaction induced by the treated serum involved the monocytes. After thirty-three of the thirty-nine injections there was a marked initial fall in monocytes, averaging 69.8 per cent, with a mean of 69.2. Four of the rabbits showed a marked initial rise in monocytes, averaging 65 per cent, but this was followed by an average fall of 58 per cent below the original level. Two other rabbits showed an initial rise in monocytes but with inadequate counts to bring out the possible subsequent fall.

In one of these, Rabbit R 15, the rise was 101.5 per cent, but it was the last of a series of seven injections, made when the monocytes were at a low level before the injection, namely, 1 per cent, or 124 cells per c.mm. They rose to 2 per cent, 250 cells, in an hour and a half, and no other counts were made. The second exception was in Rabbit R 72; after a first injection in this rabbit the monocytes fell to zero, at which time a second injection was made, when the monocytes rose to 2 per cent, or 114 cells, and no other count was taken. These two exceptions may thus be considered as especially interesting, indicating as they do a low level of monocytes after repeated injections of serum.

For the entire series of 39 injections, the fall in monocytes may thus be taken as about 70 per cent. The fall in monocytes was only

temporary, as will be shown in the discussion of the individual experiments and in the charts. Nevertheless, after repeated injections it will be seen that there was a general lower level in this strain of cells. There was no significant difference in percentage of the fall of the monocytes before and after infection with tuberculosis. For example, in Rabbit R 52, the monocytes fell 78 per cent with injection of 1 cc. of the serum before infection, and 79 and 87.5 per cent after the two injections of 2 cc. of the serum after infection. But, as will be noted on the charts, the rebound in monocytes after their fall was much greater in the tuberculous than in the normal animal. This can be attributed with good reason to the much greater available supply of monocytes in the tissues of the tuberculous animal.

The initial change in lymphocytes was much more irregular; after 22 injections, the lymphocytes fell an average of 45.40 per cent, while after 17 injections they rose 35.36 per cent. Moreover, there was a wide variation in the percentage of rise and fall in both series.

The neutrophilic leucocytes fell and then rose above their original level after 28 of the 39 injections. In seven instances there were not enough counts to show the rise, while in four the cells rose, but not to the original level.

These changes in the blood cells after the serum of treated and untreated roosters are shown in four charts (Charts 6 to 9).

Chart 6, Rabbit R 59, illustrates a case in which both monocytes and lymphocytes fell after the injections. In this animal the first rise in monocytes detected in the peripheral blood was to 34 per cent—3,046 per c.mm., on the morning of the 19th day after inoculation. A second count on that day, taken at 1:55 p.m., showed 25 per cent, or 2,400 monocytes per c.mm. 1 cc. of serum from Rooster R 23 was injected intravenously at 2:00 p.m. and 20 minutes later the circulating monocytes had fallen to 762 per c.mm. This was a fall of 1,638 monocytes per c.mm., or 68.25 per cent of the original number. At 4:10 the monocytes had returned to 2,641. On the following morning they were 4,026 per c.mm., which represented 33 per cent of the circulating white cells. 30 minutes after 1 cc. of serum had been given from Rooster R 24, the monocytes had fallen to 1,646 per c.mm. (59.2 per cent), but were back to 4,260 within 1 hour of the injection. A third intravenous injection of 2 cc. of the mixed sera from Roosters R 23 and R 24 was given at 11:15 a.m., with the result that the monocytes 30 minutes later had fallen to 734 per c.mm. (82.8 per cent) and at 4:00 p.m. they numbered still only 14 per cent of the total white blood cells or 1,526 per c.mm. The following morning the count of monocytes was 4,690, but 2 cc. of

serum from Rooster R 28 (11:30 a.m.) reduced this number within 30 minutes to 2,090 and within 1 hour to 674 per c.mm., or 86 per cent. The count at 3:15 p.m.—3¾ hours after the injection—showed the monocytes still only 5 per cent, 1,418 per c.mm.

As is shown in Chart 6, 1 cc. of serum in each of two instances was followed by a lowering of the monocytes 68.25 and 59.2 per cent respectively of their original

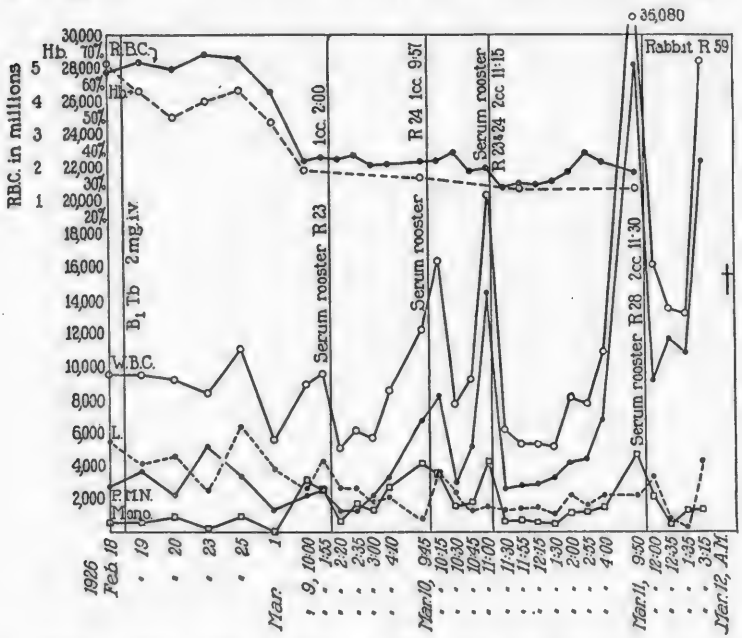

CHART 6. Blood counts of Rabbit R 59, infected with tuberculosis, show a fall of monocytes after each of four injections of rooster serum containing antibodies for rabbit monocytes.

level; in the same animal 2 cc. of these sera in one instance and 2 cc. of another similar serum at another time reduced the monocytes 82.8 and 86 per cent respectively of the original level. In both instances after the injection of 1 cc. of serum the monocytes returned within 2 hours to the level before injection; while on the other hand, with the larger doses the reduction was maintained for more than

CHART 7. Blood counts of Rabbit R 43, infected with tuberculosis, showing the contrast between the effect of rooster's serum containing antibodies to rabbit monocytes, that of the same serum after inactivation by heating, and that of normal rooster's serum.

5 hours, recovery taking place at some time during the night after the injection. The marked rise in monocytes, after the fall, in this tuberculous animal is to be compared with the smaller rebound in the normal animal illustrated on Chart 8, and especially on Chart 9. These injections were all made within a period of 3 days. The fall and rise in neutrophilic leucocytes after each injection are clear on the chart. On the day following the last observation the rabbit was found dead, and postmortem examination showed extensive tuberculosis with a terminal superimposed pneumonia, thus accounting for the rise of neutrophilic leucocytes during the last 3 days.

Chart 7, which shows the reaction after a series of injections of treated serum, of serum inactivated by heat, and of normal serum, illustrates the general findings in one of the animals in which the lymphocytes rose after the injections while the rise in neutrophilic leucocytes was relatively slight. The animal was in the phase of anemia-leucopenia from the involvement of the bone marrow (14).

It will be noted on Chart 7 that on the 14th day following inoculation with 2 mg. tubercle bacilli intravenously, the monocytes were found to have risen in the peripheral blood to 27 per cent, 1,900 per c.mm., with characteristic qualitative changes toward the epithelioid type. 1 cc. of serum from Rooster R 24 was injected intravenously at 11:30 a.m., and 15 minutes later the monocytes had fallen to 3 per cent, or 183 per c.mm., a fall of 90 per cent. In this instance the fall in relative percentage and absolute numbers of monocytes was approximately identical, that is 88 and 90 per cent respectively. There was a compensatory rebound in the monocytes 2 hours and 45 minutes later to 38 per cent, 4,080 per c.mm.

The following morning the monocytes were 25 per cent, 2,050 per c.mm., that is to say, about at the level observed 23 hours earlier. The injection of 1 cc. of the same serum used the previous day, which had, however, been heated to 60°C. for 1 hour in a water bath, was followed by a definite rise in monocytes. The percentage and total counts of these cells 15 minutes, ¾ hour, 1½ hours, and 3 hours following the injection were 30, 25, 26, and 38 per cent, and 2,665, 2,260, 2,260, and 3,845 cells per c.mm. respectively. Thus there was a rise in monocytes ranging from 30 to 87.5 per cent. The contrast between the reaction to the heated and unheated serum is well illustrated in the chart. At 1:30 p.m. of the same day 1 cc. of the same serum unheated was given intravenously and within 15 minutes the monocytes had fallen from the last figure noted above to 17 per cent, 1,612 per c.mm. and to 1,415, 30 minutes later, from which level there was a gradual recovery during the remainder of the afternoon. On January 21, the 3rd day of this series of observations, 1 cc. of fresh, normal rooster's serum (R 25) was injected intravenously, and caused no more fluctuation in the cells than followed injection of the heated serum from the treated rooster. The percentages of monocytes during the day were, respectively, 35, 29, 40, 37, 33, 38, and 30 per cent; and the actual cell numbers 3,700, 3,190, 5,002, 3,745, 3,235, 3,542, and 3,770 per c.mm. The day after these results the serum of Rooster

R 24 again caused a depression of the monocytes, 0.8 cc. injected intravenously lowering the level from 35 per cent to 13 per cent, 2,800 to 1,134. 4 days later, January 26, a final test was made in this animal, 1 cc. of serum from another treated rooster (R 28) lowering the monocytes from 47 per cent (4,248 per c.mm.) to 8 per cent (736 per c.mm.) a fall of 82.7 per cent, and with the exception of one count of 2,632 the trend continued downwards to the low figure of 4 per cent or 214 cells per c.mm. (a fall of 94.96 per cent) 2½ hours after the serum was administered. On the morning of the 27th of January, the animal was found dead. The lungs showed some tubercles and congestion but no consolidation.

On finding that treated serum could be inactivated by heating at 60°C. for 1 hour, reactivation was attempted by the addition of a small amount of fresh normal rooster's serum. A second rabbit was given an injection of inactivated serum, followed by an injection of heated serum plus normal rooster's serum. The experiment was as follows:

The tuberculous rabbit, R 46, was given 3 cc. of treated serum from Rooster R 24 at 12:25 p.m., March 29, when the monocytes were 29 per cent, 3,350 per c.mm. An immediate fall in monocytes with rise in lymphocytes was noted, the low point for monocytes—2 per cent or 175 per c.mm., or a fall of 95 per cent—being reached 3 hours after the injection. The following morning, March 30, 3 cc. of the same serum inactivated at 60°C. for 1 hour were given, the monocytes numbering 18 per cent or 1,467 just before the injection, and 1 hour later they were 30 per cent or 1,875, a rise of 27.81 per cent. During the remainder of the day they were found to represent 20, 26, and 19 per cent of the white blood cells. On the 3rd day, March 31, the rabbit was injected with 3 cc. of the inactivated serum from Rooster R 24, to which had been added 1.5 cc. of fresh, normal serum from Rooster R 21. The monocytes at 9:10 a.m. just previous to the injection were 4,200 per c.mm. (58 per cent), 1 hour later 4,990 (59 per cent), and 4½ hours after 2,062 (33 per cent), with a return to the previous level of 4,580 monocytes per c.mm. at 4:40 p.m. While the reaction was somewhat delayed and not so great in degree as that observed with unheated treated serum 2 days earlier, it nevertheless seemed definite and similar in kind.

We assume on the basis of such results that the serum from roosters subjected to repeated intraperitoneal injections of emulsions of lungs from rabbits with massive tuberculous consolidation causes a greater decrease in the number of circulating monocytes in normal and tuberculous rabbits (70 per cent) than is produced by normal rooster's serum (53 per cent). The serum loses this property on heating, but it can be at least partially reactivated by the addition of normal rooster's serum.

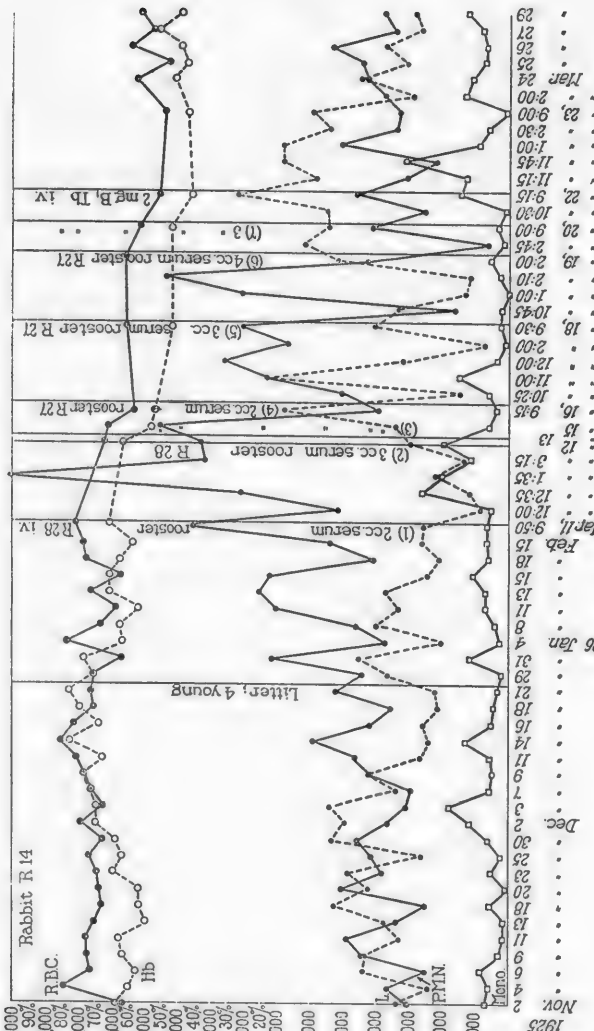

CHART 8—*Continued on Next Page*

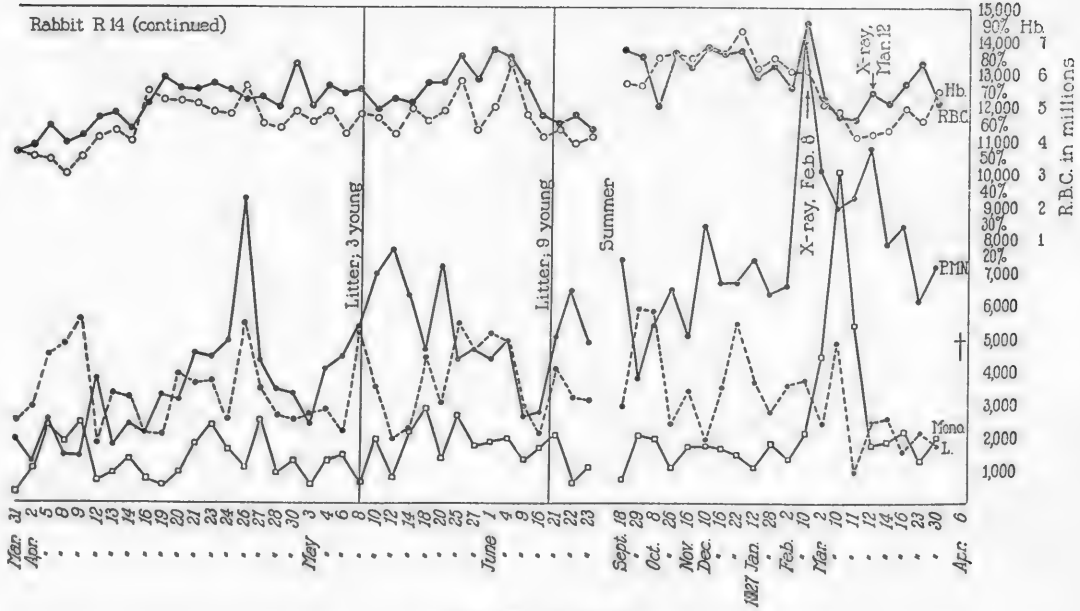

CHART 8—*Concluded*

CHART 8. Blood counts of Rabbit R 14, showing the effect of rooster's serum containing antibodies to rabbit monocytes, given before infection. During a long period with favorable M/L index the infection was quiescent but there probably occurred a rupture of a tubercle into the blood stream about February 10, 1927, with subsequent reversal of the M/L index correlated with a generalized miliary tuberculosis. Survival 380 days.

In the three animals, R 77, R 78, and R 79, which received the largest doses of the serum, namely, 10 cc., blood counts were not made until the next day. In three instances the monocytes fell, in two they rose, and in the other instance the animal died immediately after the second injection.

Following this series of observations, a limited number of rabbits were given treated sera of the above type, both before and after injection with tubercle bacilli. Charts 8 and 9, of Rabbits R 14 and R 72, may be taken as representative of this group of experiments.

During a control period for Rabbit R 14 of more than 4 months, which included one pregnancy, observations were made repeatedly on red blood cells, hemoglobin, and the different types of white blood cells (Chart 8). Between March 11 and 22, 1926, this rabbit received seven intravenous injections of antimonocytic serum from Roosters R 27 and R 28, 20 cc. in all. The lymphocytes rose to a higher level than ever observed previously in this animal, 8,200 per c.mm., and at the same time the monocytes showed a fall in total numbers per c.mm. of blood, the M/L ratio changing from 0.250 to 0.181. It will be noted on the chart that the monocytes were maintained at a low level during the latter part of the period of injection, March 16 to 22. On March 22, 2 mg. of Strain B-1 tubercle bacilli were given intravenously. The progress of the disease can be followed on the chart. The number of red cells and percentage of hemoglobin, which were already low due to the moderate hemolytic action of the treated serum, fell no lower after the inoculation; but about the 11th day a leucopenia of the neutrophilic leucocytes occurred together with a moderate transitory rise in the monocytes and lymphocytes, changes indicative of a mild reaction to the infection. A good resistance was shown by the general rise in the red blood cells and neutrophils, and on the 28th day after infection by eosinophils reappearing in the peripheral blood. The animal had two pregnancies, with litters of three and nine respectively, after infection. The monocyte-lymphocyte ratio fluctuated somewhat and the monocytes remained between one and two thousand as contrasted with the pre-infection level which averaged well below 1,000; otherwise there was no evidence of progressive disease. The weight was well maintained and there was no fever. On February 2, 1927, the blood showed neutrophilic leucocytes 6,525; basophils 2,176; lymphocytes 3,535; monocytes 1,360; red blood cells 5,500,000; and hemoglobin 76 per cent. It is clear that this count indicated that the animal was showing good resistance and this was confirmed by an x-ray plate. On February 8 the plate of the chest showed some haziness at the left apex and a few nodular shadows near the hilum, particularly on the right side. 2 days later, however, changes in the peripheral blood began which reached their climax on March 10 with 10,000 monocytes, 4,800 lymphocytes, and 4,700,000 red blood cells per c.mm. On March 12 another x-ray plate was taken of the chest and

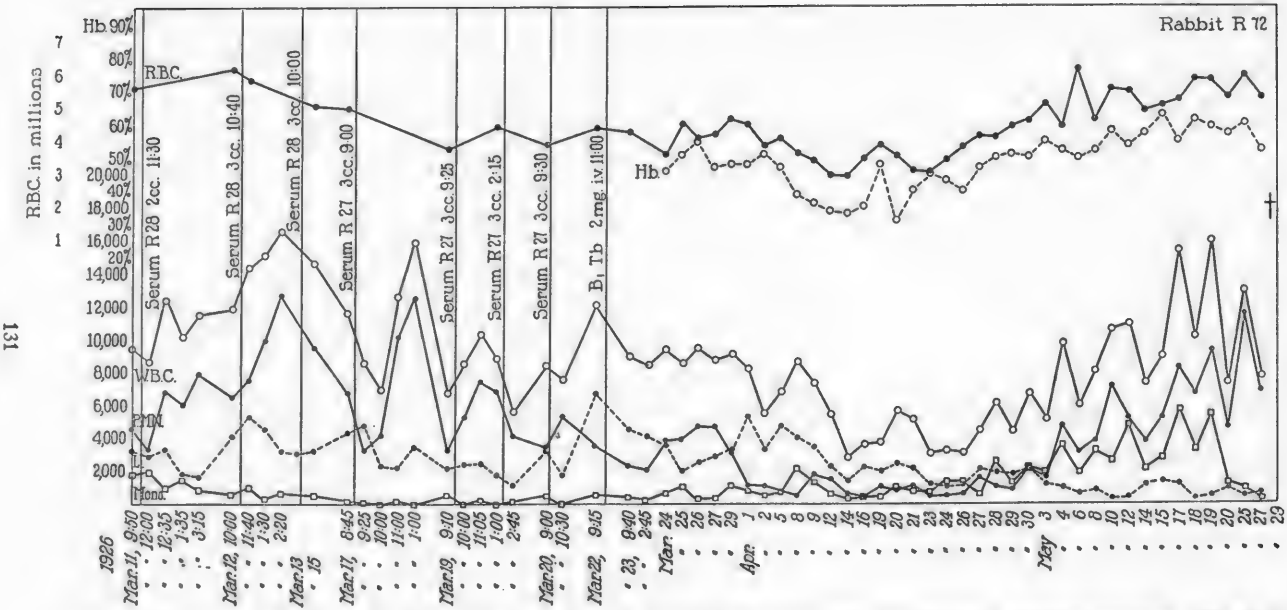

CHART 9. Blood counts of Rabbit R 72 showing the effect of rooster's serum containing antibodies to rabbit monocytes. The M/L index was relatively low before infection. Survival 68 days.

miliary shadows were found, which extended throughout the left side with total collapse of the lung on the right side, the heart being markedly displaced toward that side. There had been a gradual loss of about 700 gm. in weight since January 1. On April 6 the rabbit died, 380 days after the original infection.

At autopsy the diaphragm was found markedly lower on the right side than on the left, a spontaneous pneumothorax existing with complete collapse of the right lung in so far as fibrinous adhesions between visceral and parietal pleura would permit. The central portion of the collapsed lung contained a large cavity extending to the pleura and having a caseous wall. Numerous tubercles of various ages were scattered throughout both lungs and the kidneys contained multiple tubercles. Right humerus and femur contained single caseous areas each but no tuberculous lesions were found in the other organs. In this animal the ultimate cause of death was an acute exacerbation of a chronic, previously well controlled. tuberculous process.

Rabbit R 72 received 20 cc. of serum from Roosters R 27 and R 28 intravenously in seven injections during a period of 9 days. On Chart 9 is an excellent illustration of a sustained low level of monocytes during a series of injections of the treated serum. The monocytes which at the beginning were just under 2,000 per c.mm. were entirely lacking in six of the differential counts during the period of serum injections. The trend of the decrease is shown in Chart 9. Several counts were made each day upon which serum was administered. They revealed a transitory rise in neutrophilic leucocytes, the lymphocytes remaining within their normal limits until after the last injection. On March 22, 1926, when 2 mg. B-1 tubercle bacilli were given intravenously, the lymphocytes reached their highest number, 6,800 per c.mm., with 480 monocytes. The M/L ratio changed from 0.470 to 0.071 during the injections. The course of the disease, as indicated by the curve of the blood cells, closely paralleled that of normal stock rabbits with a naturally low M/L ratio and relatively high resistance, which were given the same dose of organisms (see (14), Chart 12 for Rabbit R 11).

A study of the data in Table XI shows that the results of the treatment with the serum were not consistent and the variations in the dosage of serum make the records of the different animals difficult to compare; however, the six animals with repeated doses (Tables IX and XI) survived 154 + days; and, if the three that received the toxic doses of 10 cc. are omitted, it will be noted that five out of eleven, or 45 per cent of the treated animals, survived beyond 70 days, while in the original control series only eight out of 50, or 16 per cent, survived that long. It is clear that with this procedure we have not induced a resistance greater than that possessed by an occasional rabbit with natural high resistance, but that we may have increased the resistance of the individual animal to some

extent. Thus a larger percentage of the rabbits treated with immunized rooster serum survived into the chronic stage of the disease than those of the untreated, control group.

The Course of Tuberculosis Following an Increase in Monocytes Experimentally Induced with Tuberculo-Phosphatide and Tuberculo-Fatty Acids

Influence of the Phosphatide.—The identification in the phosphatide fraction isolated by Anderson (32) from the tubercle bacillus of a maturation factor for monocytes and epithelioid cells (33) has made it possible to induce chemically a proliferation of these cells in experimental animals. It is significant that none of the fractions from the bacillus thus far tested have given a direct increase in lymphocytes, whence it follows that when the M/L ratio has been changed in these experiments it probably has been through an effect on the monocytes. In the experiments which follow the phosphatide and the fatty acids from the human tubercle bacillus, Strain H-37, have been used.

Analysis of the phosphatide fraction yielded a phosphorus-free liquid, saturated fatty acid, which has seemed to carry almost quantitatively the active principle for monocytes and epithelioid cells (34). With this product, as well as with A-3, it has been possible to affect by chemical means the relative and absolute concentration of the monocytic strain of cells in rabbits without other evident change in the animals. Because of the small quantities of these materials available, the number of experiments has been limited. Hence the results here described on the survival period of tuberculous rabbits treated with phosphatide A-3 or the liquid saturated fatty acid have little statistical significance. They are, nevertheless, suggestive.

Seven rabbits (see upper section of Table XII) received intraperitoneal injections of the phosphatide A-3 in doses totaling from 240 to 1,040 mg., the single dose ranging from 20 to 80 mg. The reaction of the tissue, induced by the A-3 given by this route, was roughly proportional in amount to the dose (33). Each animal received 1 mg. of bovine tubercle bacilli, Strain B-1, intravenously after the series of injections of phosphatide. Table XII indicates the dosage of the phosphatide and the period of survival of each rabbit, together with that of control rabbits receiving comparable doses of bacilli from the same suspensions. It will be seen that five of the phosphatide rabbits died very quickly in from 14 to 23 days, one survived to 44 days, and the last was killed at 78 days to terminate

TABLE XII

Survival period of tuberculous rabbits treated with the phosphatide A-3 prior to the intravenous injection of 1 mg. B-1 tb.								Controls receiving no phosphatide					
Number	Dosage of A-3 intraperitoneally	M/L index			Died	Killed	Tuberculosis post mortem	Number of specific control	M/L index		Died	Killed	Tuberculosis post mortem
		Before A-3	Before tbc. After A-3	Of last count before death					Before tbc.	Of last count before death			
					days	*days*					*days*	*days*	
R 361	1,040 mg. in 13 doses	0.313	0.430	1.143	17	—	++++ acute	R 411	0.216	1.530	31	—	+++ acute
R 362	1,040 " " 13 "	0.296	1.428	0.621	20	—	++++ "	R 412	0.186	1.066	—	120	+++ chronic
R 461	560 " " 7 "	0.192	1.375	0.514	44	—	++++ "	R 475	0.263	0.300	—	78	++++ "
R 464	560 " " 7 "	0.083	0.424	0.300	—	78	++ chronic	R 477	0.350	0.576	—	78	+++ "
R 472	240 " " 3 "	0.350	0.391	2.643	21	—	++++ acute	R 476	0.071	0.388	26	—	++++ acute
R 481	300 " " 15 "	0.384	0.227	0.944	23	—	++++ "	R 512	0.093	2.615	—	63	++ chronic
R 483	300 " " 15 "	0.194	0.290	0.382	14	—	++ "	R 515	0.130	0.182	31	—	++++ acute
R 479	150 " " 15 "	0.416	0.241	0.880	—	63	+++ chronic	R 513	0.151	0.935	—	63	+ chronic
R 480	150 " " 15 "	0.238	0.476	0.400	—	63	+++ "	R 514	0.111	0.355	—	63	+ "

the experiment. Of the controls, three died spontaneously in 26, 31, and 31 days, the remaining four surviving until killed 63 to 120 days following infection. That is to say, the average length of survival of the seven tuberculous rabbits previously treated with the phosphatide fraction from the human tubercle bacilli was 31 days, only one of the seven animals failing to succumb spontaneously under 78 days. On the other hand, four of the seven control rabbits receiving the same dosage of the same suspension of organisms survived beyond 31 days and were killed in from 63 to 120 days to complete the experiment, thus making the average period of survival in this group 61+ days.

Two rabbits (R 479, R 480, lower section of Table XII), which received only 150 mg. of the phosphatide in fifteen divided doses of 10 mg. each, survived to 63 days following 1 mg. B-1 tubercle bacilli, as did also their controls (R 513, R 514). All were autopsied at the end of this period. The rabbits receiving the phosphatide showed a more extensive pulmonary tuberculosis than the controls, with marked omental, splenic, and renal tuberculosis, the controls having no lesions or only very slight ones in these organs.

It is of interest that none of the rabbits showed any hypersensitive or allergic reactions on skin testing or as a reaction to the introduction of living tubercle bacilli. Each animal had large quantities of tubercular tissue produced by tuberculo-phosphatide (33), and it is well known that any rabbit showing a similar degree of tubercle formation due to the disease itself would, upon reinfection, have died within 12 hours (35). Thus, there seems to be no connection between skin sensitiveness and allergy on the one hand and the cellular lesion *per se* characteristic of the disease on the other. A lowered resistance to the infection accompanied the presence of the chemically produced epithelioid cells, as revealed by the shorter survival and the wide dissemination of tuberculous lesions found at autopsy, as compared with the control animals.

Four rabbits, which had received thirteen intraperitoneal injections of 80 mg. each of phosphatide A-3, were separated into pairs, the first pair receiving 1 mg. bovine tubercle bacilli each intraperitoneally, the second pair the same dosage subcutaneously in the right groin. It will be seen from Table XIII that in these particular rabbits, chosen without reference to the blood picture, the M/L index following injection of the phosphatide was considerably lower than that noted in other animals of the same series, a condition probably indicative of more than average resistance. The four control animals, likewise, showed, with one exception, a low pre-infection level of monocytes. All eight rabbits survived without the development in the peripheral blood of the anemia and leucopenia, which in our experience has followed the intravenous inoculation with this strain of

TABLE XIII

Rabbits receiving phosphatide A-3 before infection *via* the intraperitoneal and subcutaneous routes								The corresponding controls					
Rabbit	Phosphatide A-3, H-37	M/L index		Dosage and route of tb.	M/L index	Survival after infection	Tuberculosis post mortem	Rabbit	Dosage and route of tb.	M/L index		Survival after infection	Tuberculosis post mortem
		Before A-3	After A-3		Last count before death					Before infection	After infection		
R 363	1,040 mg. intraperitoneally (13 doses)	0.113	0.320	1 mg. B-1 intraperitoneally	0.040	K. 130 days	No gross lesions	R 413	1 mg. B-1 intraperitoneally	0.250	1.285	K. 120 days	Extensive chronic lesions
R 364	" "	0.205	0.282	" "	0.263	" "	Omentum, liver, small amount tuberculosis	R 414	" "	0.105	0.324	K. 130 days	Few scattered chronic foci
R 365	" "	0.172	0.200	1 mg. B-1 subcutaneously	0.095	K. 131 days	No gross lesions	R 415	1 mg. B-1 subcutaneously	0.143	0.382	K. 133 days	Small local focus in groin only
R 366	" "	0.346	0.593	" "	0.227	K. 133 days	Right groin and apex of lung tuberculous	R 416	" "	0.562	0.340	" "	Small local focus in groin

organisms. They were killed for autopsy during a period of 120 to 133 days after infection. Of those receiving phosphatide the two rabbits (R 364 and R 366) with higher M/L indices were the only ones to show any gross evidence of tuberculosis post mortem, and in the control group the only rabbit (R 413) showing marked chronic lesions had a pre-terminal M/L index above 1. Thus the ratio of monocytes to lymphocytes in the blood before death reflected the extent of tuberculous involvement as found at autopsy in this group of rabbits.

An enhanced resistance to infection *via* the peritoneal cavity after local cellular stimulation has been demonstrated repeatedly and the

TABLE XIV

Course of Tuberculosis in Guinea Pigs Treated Intraperitoneally with the Phosphatide A-3 Prior to Infection

Guinea pig	Dosage of A-3 intraperitoneally	M/L index before infection		H-37 tb.	M/L index	Survival after infection	Tuberculosis post mortem
		Before phosphatide A-3	After A-3		Last count before death		
R 467	280 mg. in 7 doses	0.280	0.750	0.03 mg. intraperitoneally	1.170	K. 74 days	+++
R 468	" "	0.083	0.500	" "	—	" 74 "	+++
R 469	" "	0.140	0.900	" "	—	D. 70 "	++++
R 470	" "	0.260	0.416	" "	0.857	K. 74 "	++++

Three of the specific controls in this experiment had M/L indices before infection of 0.040, 0.041, and 0.184, but incomplete blood studies make further analyses of the cells of the peripheral blood in this group impossible. The tuberculous lesions found post mortem, however, in the guinea pigs that had previously received the phosphatide were as much more extensive than those found in the controls as the pre-infection M/L indices in the former were higher than those in the controls.

fact has been emphasized recently by Gay in his De Lamar lecture on "The fundamental factors of immunity" (36). Whether the general cellular reaction within the peritoneal cavity to the phosphatide fraction provides sufficient non-epithelioid cell-types to care for the infection, or whether the monocytic strain itself, or other factors are involved in resistance under these conditions are questions which cannot be answered at this time. Certainly the portal of entry for the organisms is a very important factor in the ultimate development and handling of the disease by rabbits.

Four guinea pigs (R 467 to R 470) each received seven intraperitoneal injections of the phosphatide A-3, H-37 (40 mg. per dose) preliminary to infection by the same route with 0.03 mg. living human tubercle bacilli, Strain H-37 (Table XIV). As in the rabbits previously subjected to the same treatment, each guinea pig showed a considerably higher M/L index after the tuberculo-phosphatide than before, and the two (R 467 and R 470) whose blood counts were obtained just before autopsy showed the progressive nature of this change during the course of the disease. Unfortunately incomplete blood studies in this series with its controls necessitate dependence upon postmortem findings for the analysis of any possible influence of the lipoid on the progress of the disease.

The first guinea pig to succumb was one of the control animals (R 486) which died 47 days after infection with marked involvement of liver and spleen, enlargement of the superficial lymph nodes, and an occasional pulmonary tubercle. Guinea Pig R 469, which had shown an original M/L index of 0.140 with a marked increase up to 0.900 after the phosphatide injections, died 70 days after infection, with the most extreme generalized tuberculosis we have ever seen in this species, including involvement of omentum, liver, spleen, testes, lungs, bone marrow, superficial and deep lymph nodes. The remaining three guinea pigs which received the phosphatide were autopsied at 74 days and their three controls at 74 and 82 days to end the experiment. In every instance the extent of the tuberculous involvement in the phosphatide guinea pigs greatly exceeded that found in the control animals. The omentum was massively involved in the first group and not at all in the last; liver, spleen, and lymph nodes were uniformly more extensively involved in the animals which received the phosphatide than in control animals with which direct comparison was made.

Our evidence on resistance to intraperitoneal infection with tubercle bacilli, as influenced by the intraperitoneal injection of phosphatide, is somewhat conflicting in the rabbit and guinea pig, and with the two acid-fast strains of organisms, bovine and human. To the bovine strain resistance was apparently increased; while to the human it was certainly not increased and perhaps was lowered to some degree. Further studies are being made.

Influence of the Liquid Saturated Fatty Acid.—Inasmuch as the principle inducing proliferation of epithelioid cells is apparently contained almost quantitatively in the liquid saturated fatty acid derived from the phosphatide, we should expect it to influence the susceptibility of the body to tuberculous infection as does this latter. This has proved to be the case. An increased M/L index in the blood, induced by the fatty acid, has preceded rapidly progressive, fulminant disease when tubercle bacilli were introduced.

In two rabbits (R 500 and R 501) the normal M/L index rose after a total intraperitoneal dosage of 192 mg. of fatty acid in 6 cc. of mineral oil (Table XV), in one instance from 0.017 to 0.250, and in the other from 0.400 to 0.500. In

TABLE XV

Course of Tuberculosis in Rabbits Receiving Intraperitoneal and Intratracheal Injections of Fatty Acid from the Tuberculo-Phosphatide A-3, Prior to Infection

Rabbit	Fatty acid from H-37 phosphatide A-3	M/L index		Dosage and route of tb.	M/L index	Survival after infection	Tuberculosis post mortem
		Before fatty acid	After fatty acid		Before death		
R 500	12 intraperitoneal injections (16 mg.) in 0.5 cc. mineral oil	0.017	0.250	1 mg. B-1 intravenously	0.276	K. 52 days	Mild chronic lesions
R 501	" "	0.400	0.500	" "	0.818	" 52 "	Extreme pulmonary and renal lesions
R 527	32 mg. in 1 cc. mineral oil intratracheally	0.888	0.322	" "	0.516	D. 21 "	Miliary tbc.
R 529	" "	0.191	0.706	" "	2.000	" 50 "	Extreme generalized tbc.
R 531	1 cc. mineral oil intratracheally	0.440	0.185	" "	0.500	" 23 "	Marked pulmonary tbc.
R 532	" "	0.292	0.461	" "	0.500	K. 50 "	Moderately extensive generalized tbc.
R 537	Control	0.430	—	" "	1.625	D. 30 "	Extensive pulmonary tbc.
R 538	"	0.276	—	" "	0.270	K. 50 "	Small pulmonary tubercles

five additional rabbits receiving varying dosages, the M/L ratio likewise rose, as for example from 0.238 to 0.740 (R 613), and from 0.080 to 0.600 (R 620). These rabbits were sacrificed for studies of the tissue reactions (37). The quantitative tissue reaction of different animals to the same dosage of fatty acid varied,

and in general was reflected in the blood by a greater or smaller number of monocytes, as had been noted previously with the phosphatide; in addition, of course, there were definite quantitative differences in the reaction directly proportional to the dose, when widely varying amounts of the material were injected. 52 days after infection with bovine tubercle bacilli the relative amounts of tuberculous disease found at autopsy in Rabbits R 500 and R 501 were directly proportional to the pre-infection M/L index and to the index shown by the blood cells just before death.

When the intratracheal injection of 32 mg. of fatty acid in mineral oil was followed by intravenous infection with bovine tubercle bacilli, death ensued in two control rabbits (R 531 and R 537), almost as soon as in Rabbit R 527. The remaining rabbit (R 529), which had received the intratracheal injection of fatty acid, died at 50 days with an M/L index of 2.00, while the two controls killed at the same time for a direct comparison had indices of 0.500 and 0.270, respectively, the amount of tuberculous involvement in each instance being directly proportional to these ratios.

The attempt to elucidate further the mechanism of varying resistance in these and other complex reactions found in tuberculosis has led us to still another series of experiments, immunological, and only indirectly cellular in character.

The Course of Tuberculous Infection in Rabbits Receiving the Tuberculo-Phosphatide Intravenously in Antigenic Doses and in Rabbits Treated with Antiphosphatide Serum

It was first shown by Pinner (38) that the Anderson phosphatide A-3 acts as a complete antigen when given intravenously in animals, the serum developing specific antibodies as indicated by precipitin and complement fixation tests. One of us (39) has confirmed these findings as regards precipitin tests, and some experimental and clinical studies have been reported, based on this property of the phosphatide (40). The salts of the fatty acids appear not to possess antigenic properties.

Two different reactions to the phosphatide have been demonstrated. Injected directly into the tissues, it induces the formation of epithelioid cells; injected into the blood stream, it gives rise to antibodies in certain animals. If these lipoid antibodies could be shown to limit the new formation of epithelioid cells in the disease through the binding of phosphatide set free from disintegrating bacilli, and, granted the epithelioid cells harbor the organisms in a host-parasite

relationship thus preventing their destruction by other phagocytic cells in the body, those individuals capable of producing the maximum concentration of antibodies to the phosphatide should have the

TABLE XVI

M/L Index in Rabbits before and after Intravenous Antigenic Doses of the Phosphatide A-3 and the Sodium and Potassium Salts of Its Fatty Acid

Rabbit	Dosage and interval of intravenous injections phosphatide A-3	M/L index		Rabbit	Dosage and interval of intravenous injections	M/L index	
		Before A-3	12 days after last intravenous injection			Before A-3	12 days after last intravenous injection
R 682	8 doses of 25 mg. q. 4 days plus horse serum	0.181	0.528	R 882	3 doses of 20 mg. q. 5 days	0.269	0.143
				R 883	" "	0.473	0.080
R 683	" "	0.216	0.125	R 884	" "	0.391	0.391
R 694	8 doses of 25 mg. q. 4 days without horse serum	0.170	0.228	R 885	" "	0.200	0.310
				R 886	" "	0.320	1.411
				R 891	" "	0.225	0.333
R 695	" "	0.180	0.093	R 892	" "	0.130	0.277
R 802	6 doses of 20 mg. q. 4 days plus horse serum	0.216	0.114	R 801	3 doses of 10 mg. q. 5 days	0.250	0.263
				R 807	" "	0.055	0.063
R 808	" "	0.163	0.203	R 815	" "	0.140	0.170
R 809	3 doses of 20 mg. q. 4 days	0.273	0.241	R 816	" "	0.273	0.285
R 817	3 doses of 20 mg. q. 5 days	0.345	0.473	R 696	6 doses of 10 mg. of K salt of fatty acid q. 4 days	0.384	0.200
R 823	" "	0.321	0.061				
R 838	" "	0.381	0.166	R 697	" "	0.250	0.122
R 839	" "	0.152	0.333	R 698	6 doses of 10 mg. of Na salt of fatty acid q. 4 days	0.120	0.166
R 845	" "	0.375	0.175				
R 846	" "	0.242	0.161				
R 880	" "	0.161	0.200	R 699	" "	0.143	0.214
R 881	" "	0.590	0.300				

greatest resistance to the spread of the disease. To test these hypotheses the following experiments were devised.

In some preliminary observations the phosphatide alone and in combination with horse serum, the sodium and potassium salts of the fatty acid from the

phosphatide (Table XVI), horse serum alone, the protein 304, and the tuberculo-polysaccharide were each given intravenously in comparable antigenic doses to rabbits. The rabbit sera were tested for antibodies, and then 1 mg. of tubercle bacilli was given each animal intravenously. Only the phosphatide and the horse serum induced demonstrable antibodies. After 50 days, 30 per cent only of the controls, 50 per cent of all treated rabbits, and 100 per cent of those animals having received phosphatide A-3 in antigenic doses were still living. With the phosphatide alone, survival was 109 days; with it in combination with horse serum, 53 days; with the sodium and potassium salts of the fatty acid, 60 and 50 days respectively; with horse serum alone, 18 days; with protein 304, 104 days; with the polysaccharide, 23 days, while the average in eleven controls was 55 days, and the range 14 to 153 days. The chief gain from these observations was the determination of a precipitin titer demonstrable in the blood of rabbits receiving phosphatide in antigenic doses, and the obvious conclusion that smaller infecting doses of bacilli must be given to determine relative degrees of protection.

Next, thirty rabbits were divided into three groups of ten each (Table XVII), which in turn were subdivided into two, the first five rabbits in each of the three major groups receiving 0.50 mg. B-1 tubercle bacilli intravenously, the remainder 0.75 mg. During the first 6 weeks following infection the rabbits comprising the first group of ten (R 849 to R 858) were given daily injections of 3 cc. each of serum from rabbits previously treated with the phosphatide in antigenic doses and shown to possess an increased precipitin reaction to the antigen (Chart 10). The effects of the administration of this serum on longevity are recorded in Table XVII.

The ten animals in the second group (R 859 to R 868) each received, instead of antiphosphatide serum, 20 mg. of the phosphatide A-3 on three occasions at intervals of 5 days, the first injection being given on the day following the inoculation with tubercle bacilli. Rabbits R 811, R 818, and R 824 also received A-3.

The third group served as controls, five receiving 0.50 and five 0.75 mg. of tubercle bacilli. Thirty-three of the rabbits were inoculated at one time from the same suspension of organisms; R 784 and R 785, from another suspension.

As a source of the so-called antiphosphatide serum, eight groups of four rabbits each were started at intervals of 5 days. Each group received a series of three intravenous injections of phosphatide. The injections, of 10 to 20 mg., were made every 5th day in 2.5 cc. distilled water plus 2.5 cc. horse serum and the blood after a preliminary titration was taken from the carotid artery by cannula on the 12th day following the last injection. The sera from each group were handled with aseptic precautions, sealed in sterile ampoules as collected, and kept in the ice box without preservative during the 5 days required for the administration of each lot. A fresh supply of serum for use in treatment was thus available every 5 days. The M/L indices before and after the intravenous injections of the phosphatide in these groups of rabbits can be seen in Table XVI. Either there was very little change, or, as in many instances, there de-

TABLE XVII

Tuberculosis in Rabbits Receiving Antiphosphatide Se and Phosp

Number	B-1 tb. intravenously	Treatment	M/L index		Survival	Tuberculosis post mortem
			Before infection	Last count before death		
	mg.				*days*	
R 849	0.50	Daily 3 cc. antiphosphatide serum (6 wks.)	0.078	0.391	*K. 34	++
R 850	0.50	" "	0.080	2.308	D. 105	+++ chronic
R 851	0.50	" "	0.150	0.700	" 98	++++ "
R 852	0.50	" "	0.208	0.440	" 165	+++ "
R 853	0.50	" "	0.160	0.500	" 108	++ "
Ave. 4..	0.50	Daily 3 cc. antiphosphatide serum (6 wks.)	0.149	0.987	D. 119	
R 854	0.75	Daily 3 cc. antiphosphatide serum	1.000	0.824	D. 14	+++ acute
R 855	0.75	" "	1.333	0.625	" 23	++++ "
R 856	0.75	" "	0.036	0.104	" 15	++ convulsions
R 857	0.75	" "	0.478	1.000	" 70	++++ chronic
R 858	0.75	" "	0.500	1.000	" 21	+++ acute
Ave. 5..	0.75	Daily 3 cc. antiphosphatide serum	0.770	0.875	D. 28	
R 784†	1	Normal rabbit serum 2 cc.	0.125	1.833	D. 51	++++
R 785	1	" " 2 cc., 4 doses	0.400	0.952	" 16	+++ acute

Number	B-1 tb. intravenously	Treatment Each dose 20 mg.	M/L index	
			Before infection	Last count before death
	mg.			
R 859	0.50	Phosphatide 3 antigenic doses intravenously after tbc	0.146	1.625
R 860	0.50	" "	0.038	3.500
R 861	0.50	" "	0.351	0.700
R 862	0.50	" "	0.151	1.143
R 863	0.50	" "	0.257	0.830
Ave. 5..	0.50	Phosphatide 3 antigenic doses intravenously after tbc	0.188	1.559
R 864	0.75	Phosphatide 3 antigenic doses intravenously after tbc.	0.366	0.666
R 865	0.75	" "	0.143	7.250
R 866	0.75	" "	0.180	0.160
R 867	0.75	" "	0.174	1.500
R 868	0.75	" "	0.071	0.210
Ave. 4.	0.75	Phosphatide 3 antigenic doses intravenously after tbc.	0.215	2.394
R 811	0.75	Phosphatide 3 antigen doses before tbc.	0.266	0.555
R 818	0.75	3 doses of A-3 before and 3 after tbc.	0.200	0.454
R 824	0.75	" "	0.296	1.190

* K. Killed during an early stage of the disease and hence not included in the average.

† R 784 received 8 doses, and R 785, 4 doses.

M/L index		Survival	Tuberculosis post mortem	Number	B-1 tb. intravenously	M/L index		Survival	Tuberculosis post mortem
Before infection	Last count before death					Before infection	Last count before death		
		days			*mg.*			*days*	
0.146	1.625	165	++ chronic	R 869	0.50	0.166	0.400	162	++++ chronic
				R 870	0.50	0.412	0.580	63	++++ "
				R 871	0.50	0.133	0.250	41	+++ "
0.038	3.500	104	++++ "	R 872	0.50	0.074	0.350	63	++++ "
0.351	0.700	138	+++ "	R 873	0.50	0.326	1.545	84	++++ "
0.151	1.143	55	+++ "						
0.257	0.830	48	++++ acute						
0.188	1.559	102		Ave. 5..	0.50	0.222	0.625	82	
0.366	0.666	54	++++	R 874	0.75	0.150	1.125	62	+++ chronic
0.143	7.250	83	++++ chronic	R 875	0.75	0.080	0.200	63	++++ "
0.180	0.160	27	+++ acute	R 876	0.75	0.341	1.027	28	++++ acute
0.174	1.500	83	++++ chronic	R 877	0.75	0.520	0.400	27	++++ "
0.071	0.210	20	++ pn. acute	R 878	0.75	0.300	1.800	25	++++ "
0.215	2.394	62		Ave. 5..	0.75	0.278	0.910	41	
0.266	0.555	41	++++						
0.200	0.454	41	++++						
0.296	1.190	98	++++ chronic						

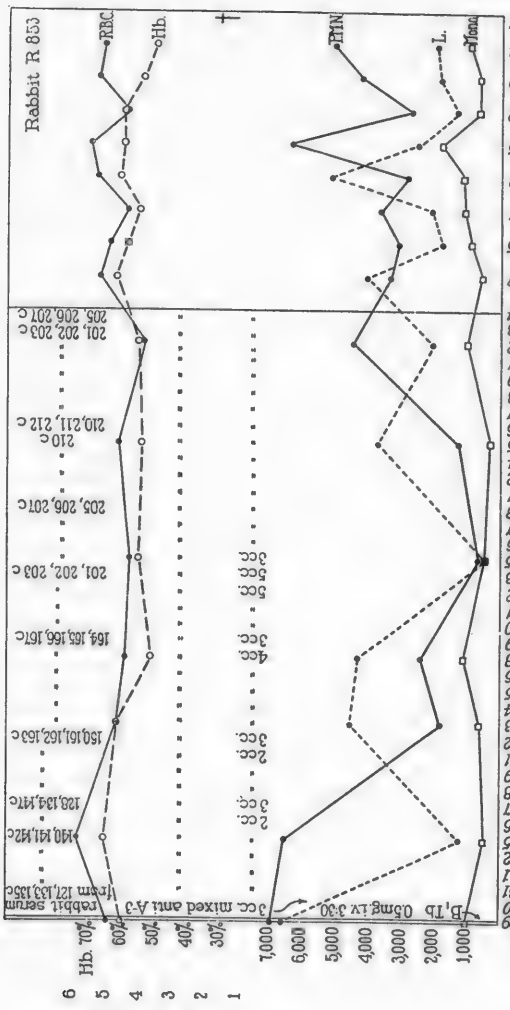

CHART 10. Blood counts of Rabbit R 853, infected with tubercle bacilli, showing the effect of rabbit's serum containing antibodies to the phosphatide A-3. A favorable M/L index, except at one point, was maintained during the period of injections, with a final rise in the index during the last month. Only a slight anemia developed as contrasted with Rabbit R 873, Chart 11. Survival 108 days.

veloped a ratio of monocytes to lymphocytes lower than obtained originally. An occasional animal showed a definitely increased M/L index with low antibody titer. An analysis of the postmortem findings in animals which received the phosphatide intravenously, together with a correlation of the serological studies in the same rabbits will be reported elsewhere.

Comparing the relative resistances (Table XVII), as evidenced by the individual survival period, in the three groups receiving 0.50 mg. of tubercle bacilli, we find that the average length of life when antiphosphatide serum was given was 119

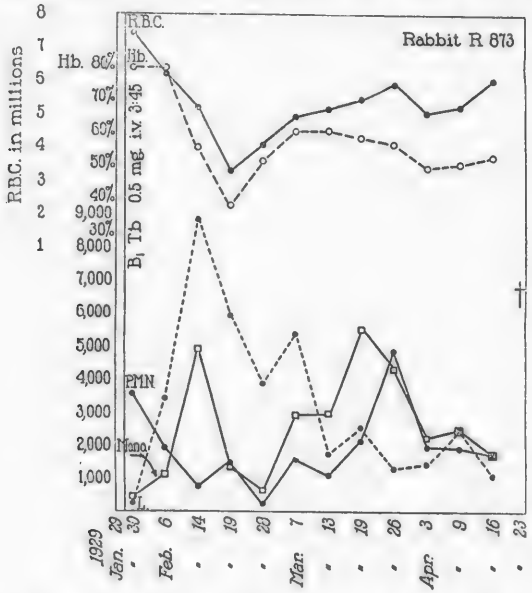

CHART 11. Blood counts of Rabbit R 873, one of the specific controls for the group of which R 853 is representative. They show the development of anemia and leucopenia, and a reversal of the M/L ratio typical of untreated tuberculosis. To be contrasted with Chart 10. Survival 84 days.

days, when the phosphatide as antigen was given, it was 102 days, and for the control group 82 days. None of the rabbits receiving the antiphosphatide serum, and only two of those getting antigenic doses of the phosphatide died under 98 days, whereas only one in the control group survived beyond this period. It is true that the first group had the lowest average pre-infection M/L index, which in itself would augur for resistance as compared with the untreated animals analyzed above, and this may explain to some extent their better showing. But

a comparison of Charts 10 and 11, the former showing the course of infection in Rabbit R 853 treated with antiphosphatide serum, the latter recording the anemia, leucopenia, and monocyte-lymphocyte fluctuations typical of the acute course of tuberculosis (R 873), will suggest that the serum treatments may have significantly altered at least the manifestations of the disease in the blood. The injection of normal rabbit serum into two animals after 1 mg. tubercle bacilli did not prolong survival.

Of the animals of Group 1 which received 0.75 mg. tubercle bacilli, two (R 854 and R 856) proved to have had cirrhosis of the liver, that in R 854 being of extreme grade. In this animal the lungs had only a moderate number of tubercles, but most of them were hemorrhagic and there was considerable blood in the air sacs around some of the tubercles. The lungs in R 856 had many tubercles, some of them exhibiting early stages of caseation. In the cirrhotic liver of this animal the tuberculosis was more extensive than is usual in this organ at this stage. The average period of survival of the three groups receiving 0.75 mg. of tubercle bacilli, namely, 28, 62, and 41 days, was relatively comparable; that is, all the animals died in the acute phase of the disease. Thus, the slight possible protection which seemed to have been associated with the use of the antiphosphatide serum and of the phosphatide in antigenic doses in the animals which received 0.50 mg. of the tubercle bacilli did not appear with the larger doses of infecting organisms.

Surviving longer than their controls, the two groups of rabbits receiving antigenic doses of phosphatide after 0.50 and 0.75 mg. tubercle bacilli lived an average of 102 and 62 days respectively. If we pair the individual animals showing closest approximation of M/L indices we have R 859 with 0.146, living 165 days after 0.50 mg. organisms, and R 865 with 0.143, living 83 days after 0.75 mg. bacilli, each having received the same antigenic injections of phosphatide. Rabbit R 861 lived 138 days after 0.50 mg. tubercle bacilli with a pre-infection M/L index of 0.351 and Rabbit R 864 lived 54 days after 0.75 mg. with an index of 0.366. These were all animals infected and then treated. It will be recalled that in our preliminary antigenic studies the survival period after 1 mg. B-1 tubercle bacilli for rabbits previously treated with intravenous phosphatide was 109 days as contrasted with 55 days for controls (page 142).

Unfortunately, the M/L indices in Table XVII calculated from the last count before death do not represent in every instance the state of the blood immediately prior to death. In certain cases they represent, because of unavoidable circumstances, counts taken some days before death. These particular indices are, therefore, not strictly comparable.

This experiment has served to emphasize again the importance of selection of stock with comparable cell indices for studies of relative immunity in tuberculosis. With this done, at least one of the variables entering into the complex responses which measure individual resistance, will have been controlled. The present observations are

merely suggestive with reference to the possible protective action of a serologic response to tuberculo-lipoid. The various serologic responses to the proteins and carbohydrates from the tubercle bacillus present closely related problems which must eventually be correlated.

DISCUSSION

The one common denominator of all these observations and experiments appears to be the relationship of monocytes to lymphocytes in the peripheral blood and tissues, the M/L index as we have termed it. It is true that no one of the experiments here recorded is conclusive, for the increase in longevity when the index has been lowered and the decrease when it has been raised have both been of slight degree; nevertheless, our results all favor the hypothesis that high monocytes and low lymphocytes are correlated with low resistance to tuberculosis.

Analysis of the records of the uncomplicated course of experimental tuberculosis in previously healthy rabbits shows that they separated into two groups with reference to the pre-infection M/L index: one in which there had been a high index, range 1.200 to 0.333, and in which all the animals showed low resistance, as evidenced by fulminating lesions and death within a brief period; and a group with from average to low indices, of which some showed high resistance (survival 145+ days) and some low resistance (survival 27 days). These data suggest that the high index may be of significance.

Our evidence has consistently indicated that during the course of the disease, on the other hand, the degree of susceptibility was marked by the promptness with which the normal M/L index was reversed and maintained at a relatively high ratio.

The experiments that followed the recognition of these facts were all devised to influence in one direction or the other the ratio of monocytes to lymphocytes in the blood, the assumption being that these changes in the blood were correlated with changes in the tissues. It was observed that whenever the number of monocytes was increased as compared with that of lymphocytes, resistance to the spread of tuberculous infection was apparently low and susceptibility great; whereas when the opposite relationship was established, instead of the acute fulminating disease associated with low resistance, we found a slowly progressing chronic tuberculosis, in which the renal lesions

shared responsibility with the pulmonary for the fatal termination. It is true that in no instance were we able to control the disease entirely, except when the subcutaneous or intraperitoneal route of infection was employed, but it should be pointed out that very large intravenous doses of tubercle bacilli were used in the experiments here reported, in order to assure a relatively brief, uniform course of the disease in the control animals. That we have been able, by means which raise the M/L index, to increase the number of animals which show the fulminant type of disease, and by those which lower it to increase the number of animals which will pass through the dangerous acute phase is presumptive evidence of a part which the cellular elements of blood and tissues play in immunity to tuberculosis. Furthermore, the fact that low resistance has been noted in the normal stock animals with naturally high M/L index tends to associate the cells with these differences. By none of the experimental procedures have we been able to do more than change the life expectancy within the limits established for the species in the control animals.

It is our belief that not only are the number of granulocytes present in the circulating blood at any moment a measure of the activity of the bone marrow and of the demand for these cells in the tissues, but that the absolute and relative numbers of lymphocytes and monocytes in the blood stream denote their absolute and relative numbers and activity in the tissues and organs of the body, thus yielding an index of the general cellular state.

At the beginning of this chapter we mentioned that the classical views on immunity in tuberculosis interpret the tubercle as the manifestation of cellular resistance on the part of the body. Evidence has, however, been presented here pointing toward the monocyte or epithelioid cell—in which the living bacillus appears to survive—as a liability rather than an asset to the defences of the body. Can this view be reconciled with the usual concept of the tubercle or is the matter too complex to be summed up in any one phrase?

It is now established that tubercles, when they have been produced by dead bacilli or by lipoids extracted from them, are innocuous and are completely resorbed. Thus the tubercle is a danger only when it contains living tubercle bacilli. It is likewise clear that only a proportion of the epithelioid cells in any infection actually contain living

bacilli. Thus the question of resistance involves at least the following factors: first the availability of epithelioid cells to contain the living bacilli, second the number and virulence of the bacilli, and third those reactions of the tissues which tend to wall off tubercles in order to check the spread of living bacilli.

It is obvious that any limitation to the spread of an infectious agent is an asset. A tubercle, when encapsulated by lymphocytes and fibrous tissue, does represent a segregation of infecting organisms. Tubercles not so limited and lesions which show diffuse infiltrations of epithelioid cells are associated with extending tuberculosis. These lesions yield shadows with indistinct margins in x-ray plates, such as are known to be associated with extending pulmonary involvement.

Our experiments have been directly concerned with the factor of the availability of epithelioid cells, but they lend support to the work of Murphy and his collaborators (16), which has well demonstrated a relationship between resistance to tuberculous disease and an artificially stimulated lymphocytosis.

Our approach to the problem of immunity to tuberculosis is a new one, or at least a different one, in so far as it has made use of an attack on the infectious agent by control of the tissue reaction. The tubercle bacillus, except in caseous areas and in the bacillemia of early or miliary infection, is to be found living within the epithelioid cell or Langhans giant cell. To strike at the organisms it has seemed desirable to work upon or through these host cells.

It has long been recognized by serologists that there exist individual differences in antibody production to the same antigen. The cellular responses to tissue irritants also show differences, and these may be indicative of differences in susceptibility to certain diseases in which the proliferation of cells is an important element. Certainly when the phosphatide A-3 has been given to rabbits in dosages of approximately 1 gm., there have been differences in the amount of induced tissue reaction, in the extent to which the M/L index was influenced, and furthermore in the general course of the disease and the period of survival after infection with tubercle bacilli.

The demonstration that the phosphatide element in the lipoids from the tubercle bacillus may give rise to antibodies when injected intravenously in antigenic doses (38, 39), and that the presence of

similar antibodies is demonstrable in the actual infection (39, 40), has opened up the question of whether these serological reactions may not be connected in some way with the cellular response to tuberculosis. The intravenous injection of the phosphatide has produced a variable cellular response in the tissues. Either as a hapten linkage or as a complete antigen in itself, it may give rise to precipitin and complement-fixing antibodies which appear at the same time as a lowering of the M/L index in the blood. This is in striking contrast to the marked increase in the M/L index after phosphatide deposits in the tissues. The serologic reaction to the phosphatide seems to influence the course of the disease through an effect on the cells; the cells thus become of special importance in the particular immunity mechanism under scrutiny. The experiments indicate that a serologic neutralization of the tuberculo-lipoid, which has been found to be a powerful stimulus for monocytes, either by active or passive immunization, not only prevents or limits an increase in epithelioid cells, but apparently influences in some way the reciprocal status of monocyte and lymphocyte so that a lower M/L index is frequently observed in the blood.

These studies must be considered as only preliminary to the analysis of the cellular factors concerned with the nature of immunity. The animal may react to the phosphatide directly by the production of epithelioid cells, or it may react to the phosphatide as antigen with the formation of antiphosphatide. The course of the disease may be influenced by the type of reaction to the phosphatide whether cellular or antigenic. Our observations must be further elaborated in order to establish fully the share which these humoral and cellular reactions to the phosphatide have in the complex processes by which immunity to tuberculosis is built up. Moreover, it is clear that the relation of the phosphatide to the formation of tubercles is only one of the factors involved in this immunity.

SUMMARY

1. In rabbits receiving a standard dose of bovine tubercle bacilli, a high pre-infection monocyte-lymphocyte ratio in the circulating blood has been correlated with the development of an acute fulminating tuberculous infection; but with an average or low index, the

course of the disease has varied. Susceptibility has been further marked by the promptness with which the M/L index rose after infection and with its maintenance at a high level; resistance, on the other hand, has been evidenced by a continued low index. The M/L index is one measure of resistance to tuberculosis.

2. Some evidence has been presented to show that when monocytes, the forerunners of the epithelioid cell, are decreased through the action of an antiserum, a greater proportion of the animals survive into the chronic stage of the disease than of the controls.

3. The production of tubercular tissue in considerable quantity by injection into the tissues of the phosphatide or liquid saturated fatty acid from the tubercle bacillus does not render the animal allergic, and seems definitely to lower resistance to the disease upon subsequent infection with tuberculosis. The degree to which the tissues react specifically with the formation of new epithelioid cells is indicated by the amount of the change in the M/L index in the blood.

4. The differences observed in individual animals in the amount of tissue reaction to a given amount of phosphatide derived from the tubercle bacillus are definite and are similar to those long noted in connection with humoral antigen-antibody responses with proteins.

5. Both in tuberculosis itself and after intraperitoneal injections of the phosphatide, the relationship of monocyte to lymphocyte in the blood before death has been a measure of the extent of the epithelioid and lymphoid proliferation found at autopsy. Hence, the M/L ratio can be taken as an index of the relative abundance of these cells in the tissues.

6. Antigenic intravenous doses of the phosphatide itself, or of antiphosphatide serum, given either before or after infection, may give a slight protection to an animal if the dose of infecting organisms be not too great.

7. Taken together, the observations of the present paper implicate the monocyte and its derivative, the epithelioid cell, when harboring living bacilli, as factors in the spread of tuberculosis in the animal. The type of reaction of an animal to the lipoids of the tubercle bacillus, whether predominately cellular or humoral, may be a decisive factor in determining resistance on the one hand and susceptibility on the other.

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